The
AMERICAN JOURNAL
of
MEDICAL TECHNOLOGY

VOLUME 25 JANUARY-FEBRUARY, 1959 Number 1

MOTION STUDY APPLIED TO URINALYSIS*

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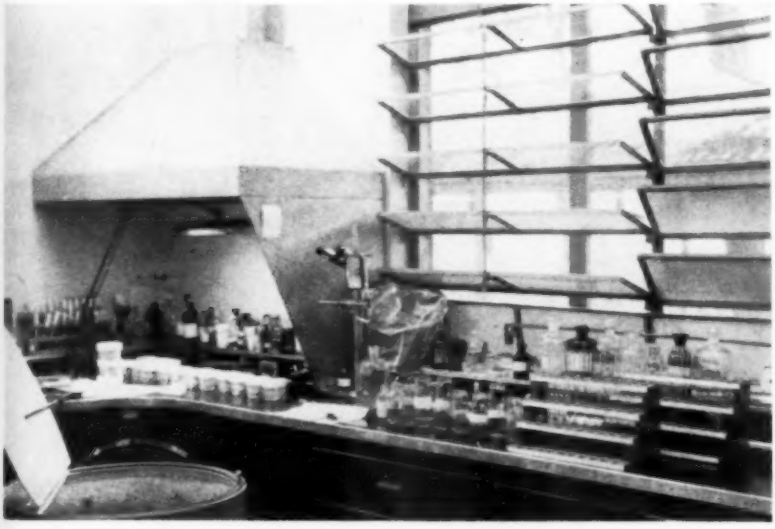
Introduction:

As the urinalysis is the most frequently requested examination in any clinical laboratory, a careful analysis is often hampered by sheer numbers alone. For this reason we have striven, through motion study,[2,6] to minimize handling and to streamline analyses. The work has been divided between technologist and laboratory assistant in a way that eliminates



Work Area

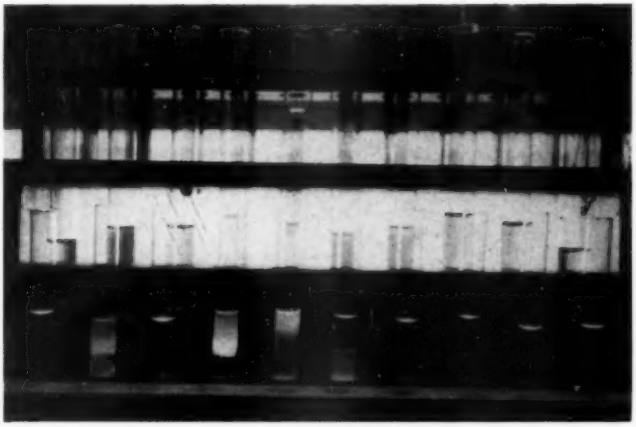
* 3rd SPF Award on Educational or Procedural Techniques. Read before the 26th Annual Convention of ASMT, Milwaukee, Wisconsin, June, 1958.

fatigue without dividing the analysis into an unrelated physical, chemical and microscopic examination.

Methods:

1. Special Equipment.
   a. A urinometer of special design, holding 30 ml. of urine, with a rubber tube connection on the bottom for quickly dumping the contents.
   b. Test tube racks of special design, with three rows of holes holding tubes for albumin, sugar, sediment and special tests, and with a row of depressions in front for determination of reaction.
   c. Illuminated viewer for easy reading of tests, especially albumin.
2. Reagents.
   a. Reaction—Methyl Red Solution (10 ml. alcoholic solution methyl red 1% + 20 ml. ethyl alcohol + 20 ml. distilled water).
   b. Albumin—Sulphosalicylic Acid Test.[5]

Special Urinometer

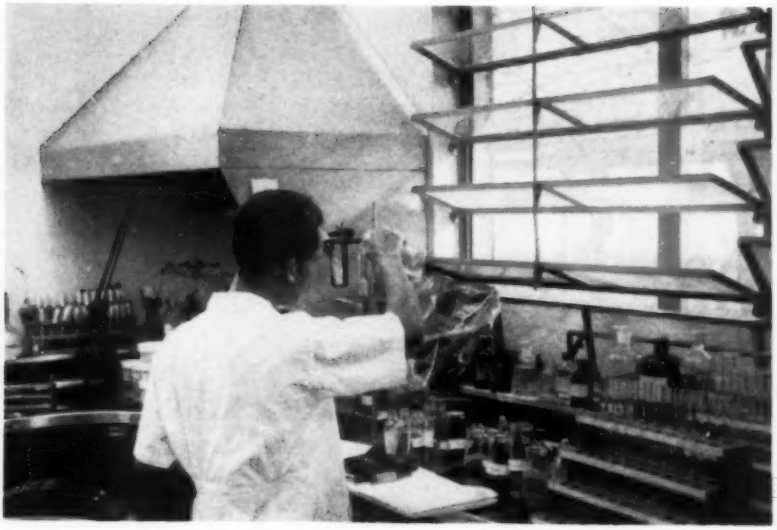


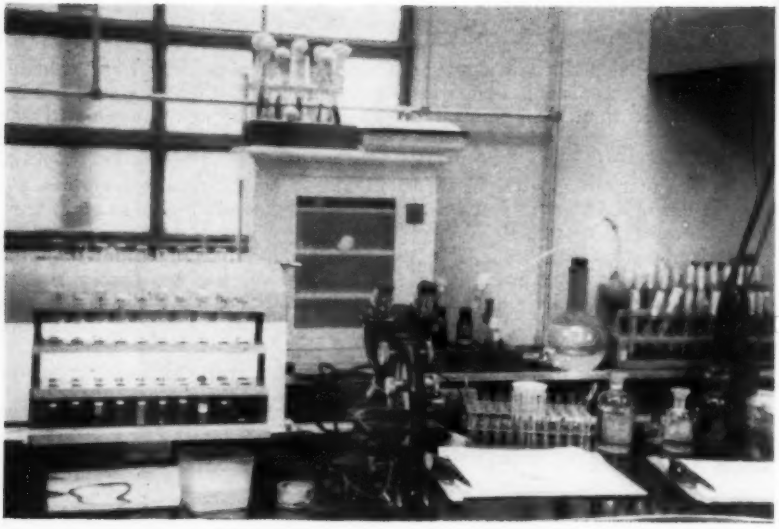
Three Row Test Tube Rack

c. Sugar—Clinitest Tablets.[4]
d. Occult blood—Occultest.[3,7]
e. Sulfa crystals—Bratton-Marshall.[1]

Procedure:

1. The secretary admits the specimens to the laboratory by giving each a number and entering the patient's name, in numerical sequence, on a work sheet.
2. The laboratory assistant then places the specimens in numerical sequence in rows of ten.
3. The special racks with appropriate tubes for ten specimens are at hand.
4. The specimen is shaken, the 15 ml. centrifuge tube filled and immediately placed in the numbered carrier of the centrifuge conveniently at hand. Without putting the specimen down, the urinometer is filled, the specific gravity read and while the urinometer is emptying, is noted on the work sheet along with the physical characteristics of the specimen.
5. The remaining portion of the specimen is placed on a laboratory cart for subsequent discard.
6. After centrifugation, as the tubes are removed, approximately 1 ml. is poured into the albumin tube (13×100 mm), the remaining supernatant poured into the sugar tube (18×150 mm), placing the last drops on the reaction spot on the front of the rack, then returning sediment tube to its place in the rack.
7. The contents of the sugar tube is poured into special-tests tube immediately behind, leaving approximately 5 drops for sugar test.
8. The reagents are now added in the following order.
   a. One drop of methyl red solution to each spot.
   b. Approximately 1 ml. 3% sulphosalicylic acid, by dropping bottle to each albumin tube.
   c. Add 10 drops of water and one Clinitest Tablet to each sugar tube.





9. The rack is then placed on illuminated viewer alongside of microscope and the technician notes chemical reactions on work sheet and proceeds with the microscopic examination of the sediment.

10. As the sediments are examined, the chemical tests may be re-evaluated at a glance; or the presence of red blood cells, sulfa crystals, etc., may be confirmed by spot tests with reagents conveniently at hand.

11. From the work sheet, the secretary types the individual reports which are signed by the technologist.

Discussion:

The taking of the specific gravity was found to be the most time consuming of all the tests. Since the volume of most specimens is way in excess of what is required for analysis, it was decided this amount could, in most cases, be discarded after the reading was taken. With this thought in mind, a urinometer was designed with a rubber tube connection on the bottom which dumped the contents at the end of each reading.

Since the centrifuge has a capacity of 64 tubes, centrifuging is done in units up to this number. Numbering the receptacles in the centrifuge in sequence of opposing pairs of carriers will enable one to centrifuge in multiples of eight specimens.

The paper strip method of reaction determination was found to be time consuming, so the spot plate method was adopted using an indicator in solution. A row of depressions attached to the test tube rack, covered with white, waterproof adhesive tape serves as a convenient spot plate.

The albumin is read as a ring test, which is easily visible against the built-in black background, as the light from the illuminating box shines

up through the tubes. Practice is needed in interpreting the degree of turbidity as even very slight reactions are readily visible.

Summary:

A system for urinalyses has been devised, minimizing lost motion and mental fatigue, thus permitting a careful, integrated analysis at a great savings of time and energy.

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THE USE OF LESTOIL FOR CLEANING OIL AND BLOOD FROM SLIDES

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Lestoil* has been found to be quite valuable in cleaning immersion oil (mineral oil base) and blood from slides. The product is economical, easily obtained, simple to use, and removes oil and blood from slides with a minimum of effort.

The method of use is as follows: The bottle cap is removed, filled with Lestoil and added to approximately 1000 ml. of water in a container (a museum jar is used in this laboratory).

When the slides are removed, no scrubbing is necessary to clean them as the slides are practically clean. Any remaining film can easily be removed by merely wiping with a cloth or sponge. The slides are then rinsed through several changes of tap water, placed in isopropyl alcohol, and dried with a clean cloth as needed.

* Lestoil-Adell Chemical Company, Holyoke, Massachusetts.

MEDICAL TECHNOLOGY IN A MID-WESTERN STATE:* KANSAS, 1957-58

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Since World War II a quiet revolution has been going on in the clinical laboratories of this country. Not only have requests for laboratory procedures doubled and quadrupled in our busy hospitals, clinics, and doctors' offices, but the list of procedures considered "routine" has expanded alarmingly and increased in complexity with each new scientific advance affecting medicine. This fantastic rise in demand for skilled laboratory service presents a real challenge to the young profession of medical technology, whose members must try to serve this demand. How are we, as a profession, meeting this challenge?

In a paper entitled, "Medical Technology: Profession vs. Occupation"[2] published in the Am. J. Med. Tech. in September, 1957, I attempted to answer this question. I expressed the opinion that we are not faring too well, particularly in the areas of public relations, recruitment, economic advancement and professional organization. Statistics gathered by various investigators[1,3] over the country indicate that the registered technologist (ASCP), voluntarily meeting the standards of the American Society of Clinical Pathologists in education and training, is in the minority as a laboratory worker, despite the obvious need for well-educated and highly trained laboratory personnel. If this trend continues, the ASCP registered technologist can expect to face increasingly serious competition from the informally trained "lab. technician" whose employment in large numbers undermines his status and threatens to destroy his professional identity. Current employment practices in many clinical laboratories, making little or no distinction between registered and unregistered personnel, have affected medical technology most adversely and made recruitment especially difficult. These effects on the profession, of course, are in addition to serious effects on the quality of laboratory service available to the patient.

But the observations and opinions expressed in this previous article were based on statistics gathered by others and dealt with professional problems on a national scale. Since then, as Chairman of the Standards and Studies Committee for the Kansas Society of Medical Technologists, it has been my good fortune to assist in making two surveys of the state of Kansas, designed to get up-to-date information about employment practices here, and opinions from individual technologists regarding the status of their profession in this mid-western state. It was surprising to find such close agreement between our conclusions and those of national investigators regarding the nature of the problems besetting medical technology, although local differences in degree were observed.

A word about the geography and economy of the state of Kansas for purposes of orientation may be helpful. Kansas is predominantly an agricultural state with most of its industry concentrated in the three largest cities: Kansas City, Wichita, and Topeka, all located in the eastern third of the state. The remainder of the state is flat prairie and communities as well as hospitals are small and widely separated. Thus,

* Received for publication May, 1958.

the scarcity of medical technologists is keenly felt in many communities in Kansas, which, because of their small size and relative isolation, have difficulty in attracting qualified personnel. In fact, the shortage of ASCP registered technologists, a serious problem nationally, is probably more sharply accentuated in Kansas than in most other states.

It would be reasonable to expect, therefore, that the ASCP registered technologist, by virtue of his scarcity as well as his superior training, would enjoy relatively higher salaries here than elsewhere in accordance with the law of supply and demand. But such is not the case. The answer to this seeming paradox lies in the widespread practice of hiring unregistered personnel to staff clinical laboratories in Kansas. This compromise with quality is dictated in part by necessity, but is due in large measure to unrealistically small wage differentials between registered and unregistered workers which discourage prospective recruits for ASCP training and are unattractive to currently registered out-of-state technologists, Kansas technologists considering a change in locality, and retired technologists wishing to return to work. We feel that low salaries and competition from informally trained personnel are our most serious problems here. It would appear, therefore, that medical technologists in Kansas face difficulties common to medical technologists nationally. We hope that this "close-up" of conditions in Kansas will be helpful to technologists in other states, serving at least as a basis for comparison.

We gathered the statistics presented here by means of two questionnaires: the first, directed to medical employers of clinical laboratory personnel in hospitals and clinics throughout the state; and the second, to individual technologists.

Table I

RESPONSE TO QUESTIONNAIRE SENT TO MEDICAL EMPLOYERS

NUMBER SENT	Replies Received	% Response—By No.	By Beds
165 (hopsitals)	106	64%	78%
88 (clinics, etc.)	39	44%	....
253 (total)	145	57%	....

Only 6 private hospitals of more than 50 beds failed to respond.

Table II

RESPONSE TO QUESTIONNAIRE SENT TO INDIVIDUAL TECHNOLOGISTS

NUMBER SENT	Replies Received	Per Cent Response
684	270	Approx. 40%

Of the 200 ASCP registered technologists listed by medical employers as current employees, 162 or 81% responded to this survey.

* * *

I. SURVEY OF MEDICAL EMPLOYERS:
Number of Workers Employed:

Hospitals.....306 full time
66 part time
Total.....372

Clinics.....133 full time
17 part time
Total.....150

Total number of employees: 522.

Registry Affiliations of Employees:

Workers were classified as registered or unregistered in 519 instances.

ASCP registrants	200	or 38%
AMT registrants	38	or 7%
Unregistered	281	or 55%

Thus, according to this survey, 62% of Kansas laboratory workers are *not* ASCP registrants.

Education of Unregistered Workers:

25% have college degrees or are specialists with advanced degrees. (Slightly more than 70% of ASCP registered technologists in Kansas have college degrees.) The rest of these unregistered workers are high school graduates, Army trained technicians, R.N.s, those with some college, and commercial school graduates.

Hours

Hospitals reported a basic work week ranging from 40 to 48 hours, not including call or overtime. Average: 42.6 hours. No data was given on total hours, including call and overtime actually worked by hospital employees.

Clinics reported a basic work week ranging from 36 to 44.5 hours and generally required no call or overtime. Average: 40.9 hours.

Call and Overtime

Medical employers stated that only 13 out of 135 laboratories do not give their employees time off or pay for call and overtime. (This is in disagreement with the statistics obtained from individual technologists. See below.)

Salaries

No distinction was made salarywise by medical employers between ASCP and AMT registrants with but two exceptions. Therefore, the two groups will be considered together.

Starting salaries for ASCP-AMT registered technologists ranged from $223 to $425 per month, with an average of $300 per month.

Starting salaries of unregistered workers, a broad classification including dishwashers as well as Ph.Ds, ranged from $110 to $650 per month, with an average of $249.00 per month.

Excluding those workers making less than $200 per month and M.A.s or Ph.Ds making over $400 per month in the "unregistered" category, the average for the 75% of workers remaining is $282 per month. (Admittedly, this is an arbitrary means of excluding workers on the extremes of the salary scale, but should give a more accurate comparison between the majority of unregistered workers and registered technologists doing the same type of general laboratory work.)

In any case, the average starting salary for the unregistered worker in Kansas, including or excluding specialists and dishwashers, is very close to the average starting salary for ASCP registered technologists, revealing a wage differential of between $18 and $51 per month.

A comparison of average wage differentials between registered and

unregistered workers on a regional basis, according to hospital size is shown in the following table:

Table III

AVERAGE STARTING SALARIES OF ASCP-AMT REGISTERED AND UNREGISTERED WORKERS BY HOSPITAL SIZE & LOCATION WITH WAGE DIFFERENTIALS

DESCRIPTION	I ASCP-AMT Reg. Techs.	II Total Unreg.	III Unreg.—75% Reporting No Aides, Specialists	WAGE DIFFERENTIALS			
				Monthly		Yearly	
				I & II	I & III	I & II	I & III
14 Hospitals in "Big Three" cities—(K.C., Wichita, Topeka)	295.00	225.50	260.00	69.50	35.00	834.00	420.00
20 Hospitals of more than 75 beds, not in big cities	298.00	246.00	280.00	52.00	18.00	624.00	216.00
28 Hospitals of less than 75 beds, not in big cities	323.00	284.00	308.00	39.00	15.00	468.00	180.00

Table III shows that in terms of economic advantage, the ASCP registered technologist receives a starting salary of at best only $834.00 more per year than the average for unregistered workers, including aides and dishwashers. As expected, all laboratory personnel are paid more in the small "one-man" hospitals than in the big city hospitals. However, the wage differentials between registered and unregistered personnel everywhere are shockingly small.

II. SURVEY OF INDIVIDUAL LABORATORY WORKERS

The majority of respondents to the questionnaire sent to individual laboratory workers were ASCP or AMT registrants (91%). Only 24 unregistered workers replied to this survey, although fewer such persons were contacted due to the fact that their names and addresses were not available. The majority of respondents were females: 236 to 34 males. More than 70% had college or graduate degrees and 49% were supervisors of departments or entire laboratories. KSMT members responded very well (66%). We believe our respondents could be described as the "cream of the crop" of technologists in Kansas.

Ages of Respondents		Ages of Active Technologists	
20-22 years	15	20-22 years	14 (93% active)
23-27 years	61	23-27 years	45 (74% ")
28-32 years	81	28-32 years	45 (55% ")
33-37 years	41	33-37 years	27 (66% ")
38 & over	68	38 & over	53 (78% ")
No age given	4	No age given	3
	270		187

The poll taken by the national ASCP Registry revealed that the largest active group was in the age range 23-27 years; 28-32 second, and 33-37 third. The Registry poll indicated that beyond the age of 37, the numbers of active technologists dropped off sharply. This does not seem

to be the case in Kansas. At any rate, more active technologists in the age range of 38 and over responded to our questionnaire.

EXPERIENCE

The average total working experience of respondents was 7½ years. Distribution was as follows:

0 years.....15	8 years.....14	16 years.....2
1 year 17	9 years.....16	17 years.....3
2 years.....26	10 years.....12	18 years.....5
3 years.....26	11 years..... 8	19 years.....2
4 years.....24	12 years..... 6	20 years.....3
5 years.....33	13 years..... 8	20-30 years.....6
6 years.....15	14 years..... 3	Over 30 years.2
7 years.....15	15 years..... 8	(One omission)

The Registry survey in 1953 revealed that the largest number of active technologists was in the group with 3 years' experience with a steady and continual drop beyond 4 years. Our largest group had 5 years' experience, with an average of 7½ years as noted above. Average length of service *on the same job* for our respondents was 4 years.

Type of Employment

The majority of our respondents work in hospitals (68%), with clinics and doctors' offices accounting for 24% (12% each). A few work in public health laboratories, research laboratories, etc., (8%).

Hours

The average basic work week reported by our respondents, not counting call and overtime, was 43 hours. (This corresponds closely to the average listed by hospital employers of 42.6 hours.)

Total hours actually worked per week, including call and overtime, ranged from 35 to 90 hours, with an average of 45.3 hours.

Call and Overtime

Fifty per cent of respodents stated that they did not receive either pay or time off for call and overtime. This is in contrast to the data received from medical employers, as noted previously.

Salaries

After an average of 4 years on the same job, our actively working respondents (excluding those with M.A.s and Ph.D.s) reported the following current salaries:

ASCP-AMT registrants, male: Range—$350-550 per month. Average: $451.00.

ASCP-AMT registrants, female: Range—$220-450 per month. Average: $322.00.

ASCP-AMT registrants, male and female: Average: $344.00 per month.

Unregistered workers, male and female: Average: $282.00 per month.

Comparing these current salaries with those listed by medical employers as starting salaries, it appears that an average annual raise of about $11.00 per month may be expected by ASCP-AMT registrants. It is

interesting to note that the *average* salary for ASCP-AMT male technologists is higher than the *highest* salary reported by any female registrant.

Few conclusions can be drawn from the average for the 24 unregistered workers responding, although the average of their salaries correspond very closely to salaries listed by medical employers for such personnel.

We asked for an estimate of the maximum salary attainable at the technologist's current place of employment. Many technologists did not seem to know this, but the average maximum salary attainable, when given, averaged $400.00 per month.

* * *

In addition to questions about education, hours, salaries, etc., of individual technologists, we asked these questions:

1. Do you believe that some form of universal regulation or standardization of all medical laboratory personnel, defining educational requirements, occupational training, and adequate supervision is desirable? Necessary?

Out of 266 expressing an opinion, 258 felt that some form of regulation was desirable or necessary with 149 checking "necessary." Only 8 respondents were flatly opposed to any change in our present system of voluntary control.

2. Do you think such regulation should be attempted on a state or national level?

The majority favored regulation on the national level in answer to this question.

3. Do you believe that state control of standards for medical laboratory personnel if made rigid enough so that the equivalent of ASCP training is required, and if sponsored by pathologists, local physicians, public health workers, and fellow technologists, would improve our professional standing and raise our pay levels in Kansas? Or are you opposed to any form of regulation of laboratory personnel?

Of 235 answers to this question, 192 favored state control, with 43 opposed, including 8 opposed to any attempt at standardization, and 35 favoring controls on the national level.

Replies to this section of our questionnaire represented the most interesting and probably the most significant findings of our survey. The overwhelmingly favorable response to these questions regarding some form of legal control over clinical laboratory personnel would indicate that Kansas technologists are much more receptive to the idea of "licensure" than was generally realized. Many of the voluntary comments of respondents concerned this question and while the majority evidently favored more effective means of regulation, there was little agreement on how this should be accomplished.

A study of replies to this section:

This section of the questionnaire revealing the opinions of a majority of Kansas technologists on the vital question of controls should lead to more enlightened leadership in our state society. Since attitudes are constantly changing, other state societies might do well to sound out their members by similar means on such controversial and emotionally-charged questions. As a matter of fact, the favorable opinions expressed in this survey regarding regulation of laboratory personnel

represent almost a complete about-face from opinions expressed by a majority of technologists at the K.S.M.T. convention here as recently as two years ago. Some of the voluntary comments received from respondents may indicate why this reversal of attitude has occurred.

Regarding salaries:

"In this area a reliable 16 year old can get 80¢ an hour for baby sitting and housework. There is no incentive to spend 3 years' time and thousands of dollars training for a job that will pay only $1.25 an hour."

"Our salaries are below dieticians, medical librarians, etc., and our starting pay here is the same as the pay for janitors."

Regarding the competitions for jobs posed by the unregistered worker:

"In this area we are plagued by the Nine Month Wonders getting the same salary as those with 5 years of schooling and registration. There is no longer any advantage to being ASCP registered."

"Doctors seem willing to pay for equipment but not for qualified personnel. This is false economy."

Regarding the need for higher educational standards:

"The Board of Registry of MT (ASCP) has consistently held down our requirements to two years of college work, thus advocating poorly trained personnel from within the profession itself. Most technologists already have college degrees, but official standards have lagged behind, penalizing the graduate technologist."

"The Registry must raise its standards to a degree qualification. We won't gain recognition from anyone until a degree is required. Judging from dieticians, O.T.s, P.T.s, medical librarians, etc., high standards are all that is needed. These other professions have degree requirements and receive high Civil Service ratings. Why can't we learn from them?"

Regarding the need for more publicity for our profession:

"Around here the general public thinks medical technology is an occupation learned by going to a trade school (like T.V. repair men), or one that can be learned by taking a correspondence course."

"People are well aware of the difference between a registered nurse, a practical nurse, and a nurse's aide. But in general, anyone who works in a laboratory is regarded as a technician."

Regarding control of laboratory standards:

"I guess we will have to regulate ourselves if the doctors won't recognize their own regulating body, the A.S.C.P."

"I recently broke in a commercial school graduate. . . . who was untrained, irresponsible and untrustworthy, so I feel strongly that there has to be some regulation to protect our fine field, as well as the general public."

Regarding a universally recognized regulating body for medical technology:

"We must govern ourselves in order to raise our standards and status. The x-ray technicians have their own organization and enjoy a higher

Civil Service rating than we do, and they need only high school plus two years of training."

"Technologists are failing to regulate themselves because the majority of them are uninspired. The 'big stick' is voluntary membership in A.S.M.T."

"I have been a medical technologist for 16 years, and today I can honestly say we are no further advanced professionally than we were when I first started. The years have only confirmed my opinion that we were never organized properly to begin with."

CONCLUSION

We feel that our survey of medical technology in Kansas, by means of questionnaires sent to medical employers and individual technologists, was highly successful and of great value to our state ASMT Society. Statistics from our survey were used in a liaison conference with Kansas pathologists recently to authenticate our claims that the shortage of properly trained laboratory personnel is critical in this state and that an important cause of the failure to recruit new professional members and keep old ones active is poor salaries. A proposed wage scale, differentiating between workers on the basis of education, training, registration and experience was submitted for their consideration. We plan to use these statistics in other ways in the future for the benefit of Kansas technologists. Perhaps surveys in other states would prove to be as valuable. We hope our findings will encourage other state societies to "get the facts" in their own areas, and be of some general interest to medical technologists everywhere.

* * * *

ACKNOWLEDGMENTS:

I wish to thank Miss Carolyn Collins, M.T. (ASCP) (ASMT), president of KSMT in 1957-58, for conceiving the idea of surveying our state by means of questionnaires, and for her valuable assistance in making this survey a success.

Thanks are also due Mr. Charles S. Billings, Executive Director of the Kansas Hospital Association, who contributed much time and effort to our survey of Medical Employers.

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THE SCOPE AND LIMITATIONS OF HISTOCHEMISTRY*

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I. Introduction

The aim of this paper is to present as simply and clearly as possible this complex and rapidly growing field of Biology. The understanding of the chemical organization of the body and particularly of its most minute elements is basic to the understanding of normal and abnormal structure and function, and, indeed of life itself. It has become the common frontier of Anatomy, Biochemistry, Physiology and Pathology.

Histochemistry means literally, the chemistry of tissues. Chemistry in its broadest sense includes all the physical and chemical properties and reactions of matter. The word tissue means fabric and originally referred to the various textures and weaves of materials of which the living body is composed. In modern usage it refers to the microscopic arrangement of cells and extracellular materials in the body.

The cells are the living units of structure. The extracellular materials (or intercellular substances as they are called) provide the medium in which cells live and are suported and nourished. The cells maintain and modify the intercellular substances and are, in turn, dependent on them.

The modern definition of a tissue is, then, groups of similar cells and their intercellular substances subserving similar or common functions. The tissues may be classified into 4 main types:

Table 1
THE FOUR PRIMARY TISSUES OF THE BODY

Tissue	Classification	Functional Specializations	Structural Characteristics	Distribution
I. Epithelium	Covering and lining membranes; glands	Protection absorption secretion	Cellular, contiguous cells, everywhere supported by connective tissue	Covering of body lining of organs; glands
II. Connective tissue	Circulating, cellular, fibrous, rigid	Connecting nourishing defending supporting	All derived from mesenchyme, many types of cells, separated by 2 types of I.C.S.	Throughout the body
III. Muscle tissue	Visceral, skeletal, cardiac	Conductivity contractility energy	Long fiber-shaped cells, everywhere supported by I.C.S. or C.T.	Musculature of organs; skeletal muscles; wall of heart
IV. Nervous tissue	Of central nervous system Of peripheral nervous system	Irritability conductivity secretion	Large cells with long fiber-like extensions, everywhere supported by cells	Brain spinal cord ganglia peripheral nerves

Histochemistry is, then, the study of the chemical and physical organization and reactions of the living elements and their products in these various tissues of the body.

* Read before the 26th Annual Convention of ASMT, Milwaukee, Wisconsin, June 1958.

II. The Aims of Histochemistry

The first aim of Histochemistry is to know precisely the normal variations in structure and appearance of all the varieties of living cells in the body. Such studies are called Cytology (cytos—cell, logos—knowledge). But since cells rarely exist independent of the non-living extracellular materials, histochemistry must also be concerned with the normal variations in structure and appearance of the intercellular substances and their relationships to cells as they both occur in tissues. Such studies make up the substance of Histology (Histos—tissue) (or microscopic anatomy).

The second aim of Histochemistry is to know precisely the chemistry of living matter. This is the substance of Biochemistry and Biophysics (Bios—life). But Histochemistry must go further and determine precisely the chemical composition and reactions, and the localization of these within living cells (Cytochemistry) and also in the intercellular substances.

The third aim of Histochemistry is to determine the changes in structure and chemistry of the tissues in relation to their normal functional changes (Histophysiology); and finally, to determine the changes in structure and chemistry of the tissues in relation to their abnormal function (or disease) (Histopathology).

III. Branches of Histochemistry

The techniques of Histochemistry will therefore include and combine the techniques of Cytology and Histology, Biochemistry, Physiology and Pathology. [1]Since the purpose of Cytology and Histology is the investigation of the microscopical structure of living matter, it follows that the study of the living structures of the cells must hold a paramount position in any histological inquiry. This aspect of the study of living matter is much neglected largely because the technical difficulties are, of course, very great. The ideal condition is to study the structures without removal from the body or without injury to the body. This can be done with minute transparent organisms which can be immobilized, brought into the field of the microscope, properly illuminated and observed with the high powers of the microscope. Such an intact organism may also be subjected to vital staining with non toxic chemical indicators such as neutral red and methylene blue. Considerable basic information about chemical reactions in living cells has been obtained in this way.

The only living tissues of the human body which can be studied in this way are the tissues of the eye and some parts of the skin. The microscopic study of these living tissues requires elaborate equipment for proper illumination. In most instances, therefore, this direct approach to the study of living tissues is not possible, and some compromise must be made.

The first of these compromises is the study of structures while they are still a part of the organism but are exposed to study by careful dissection, brought into the field of the microscope, and properly illuminated. Various techniques have been devised for this type of study.

[1] Bensley, R. R. and S. H. Bensley. 1938. Handbook of Histological and Cytological Technique. Chicago. University of Chicago Press (out of print).

Living tissues and organs can be studied *in situ* under the microscope when they are transilluminated with light, condensed and conducted around corners, by means of a quartz rod or tube. Living tissues *in situ* also have been studied microscopically in transparent chambers inserted in rabbits' ears, and through transparent windows inserted in the body wall. A third method of studying living tissues in the body is to transplant them to the anterior chamber of the eye where they can be studied under the microscope through the transparent cornea.

With these techniques it must be remembered that any manipulation of tissue produces changes which proceed at different rates according to the amount of injury and the sensitivity of the tissue. Moreover, the exposure of tissues to light intense enough to illuminate them for microscopic study introduces another source of injury. Therefore, tissues exposed for microscopic study are, at best, injured to some degree. Moreover, only a few qualitative chemical studies can be made on living tissues with these procedures.

Since the foregoing methods are not suitable for quantitative chemical studies a second compromise is to resort to the device of removing portions of the tissue and maintaining them in as indifferent a fluid as possible. No mounting medium is altogether free from objections but some are better than others. Best of all is the juice of the tissue itself. Next in order of preference after the tissue juice (since the latter is in general not available for the study of mammalian organs), the following may be mentioned: blood plasma, blood serum, aqueus humor from the eye, amniotic fluid, peritoneal or pleural exudate, and cerebrospinal fluid. In general it is best to obtain these from the same species from which the tissues are removed. The final resort is to balanced electrolyte solutions having about the same osmotic pressure as the tissue under consideration, having about the same elementary salt composition as the fluid in which the cells in question are normally bathed, and properly oxygenated. Labile tissues will retain their appearance for the longest periods in blood plasma and will undergo change rather rapidly in any of the balanced salt solutions.

Tissues so prepared for microscopic study, are surviving rather than living and functioning normally. Therefore chemical studies made on such fresh tissues only approximate the chemical and physical properties of living tissues. The chemical changes which occur as a result of injury to living tissues and those which take place as soon as life and function are suspended proceed rapidly at body temperature. The rate of chemical changes in fresh tissue removed from the body can therefore be retarded if the tissues are immediately chilled and maintained at a low temperature.

For classical biochemical studies relatively large quantities of tissue must be removed from the body. The tissue is then extracted with suitable reagents and the extracts are analyzed by the methods of inorganic and organic chemistry. The results of such studies yield quantitative information about the chemical composition of the tissues but tell us nothing about the localization of chemical substances and their relationships within the cells and the extracellular materials of the tissue.

For Histochemistry, then, the methods of Histology and Biochemistry must be further extended and combined. The techniques of Histochemistry have developed along three lines: microdissection, microanalytical methods and microscopic histochemistry.

Microdissection includes all the methods for physically isolating cells and parts of cells for biochemical study. Quantities of whole cells have been obtained in three ways: 1) large colonies of free living unicellular organisms are collected and then concentrated by centrifugation. 2) cells removed from the body are grown in suitable mounting media, outside the body, in large numbers. This is called tissue culture. The cells in tissue culture are suspended in the mounting medium and then concentrated by centrifugation. 3) Cellular organs and tissues removed from the body can be physically broken up by grinding them in an homogenizer or by squeezing them through bolting silk of fine mesh. The cells, freed from the supporting structures, are suspended in an indifferent medium and concentrated by centrifugation. Whole cells, collected by these methods can be subjected to biochemical analysis.

But microdissection has been carried even further. The microscope and more recently the electron microscope, reveals that the cell is made up of many different parts and structures. (Figures 1 & 2).[2]

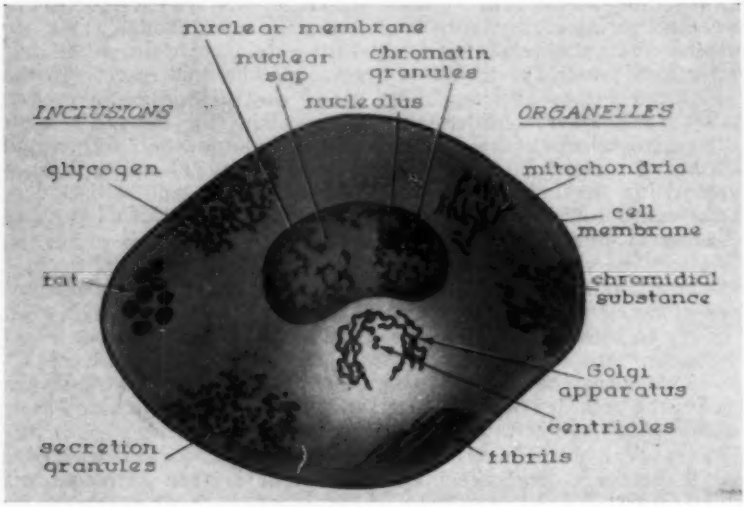

Figure 1[2]

[2], Ham. A. W., 1957. Histology 3rd. Edn. Philadelphia. Lippincott Co. p. 74, Fig. 52, p. 77, Fig. 56.

[3]The cell is, in many ways, like a pocket watch, for it runs smoothly only when all parts are properly fitted together. A watch cannot be understood by looking at its closed case or by watching the hands go round; nor can its intrinsic mechanism be understood by grinding the watch in a mortar and analyzing it for the amount of gold, iron, copper, beryllium and silica. However, the watchmaker knows each part separately; he knows that the proper combination of copper and beryllium will give the mainspring its necessary resilience, and he knows how each of the pieces fits into the whole. Similarly the cell can be understood only by knowing the structure, composition, and function of each of its component parts and by discovering their relationships to the whole.

The biochemist can tell us the substances of which cells are composed but the cytochemist, like the watchmaker, must seek to know the component parts of the cell, the localization of chemical substances

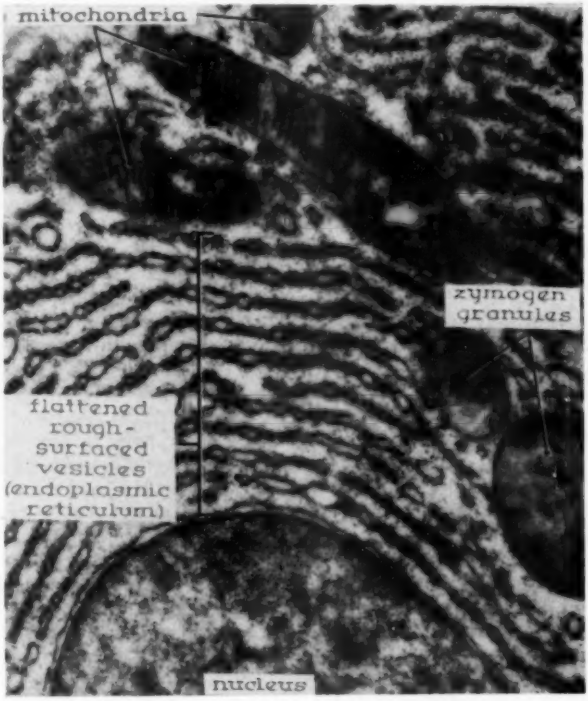

Figure 2[3]

[3] Lazarow, A. The chemical structure of cytoplasm; in Frontiers in Cytochemistry Ed. N. L. Hoerr, 1943, Lancaster, Penn., Jaques Cattell Press. (out of print).

within these components, and how they react and work together in the living cell. The first step then is to take the cell apart and to examine each component. This has been done in two ways: 1) by micromanipulation (or microsurgery) and 2) by differential centrifugation of components released from broken cells.

For micromanipulation studies individual cells are isolated and immobilized under the high power lenses of the microscope. Special glass instruments of microscopic dimensions, such as microneedles, micro knives and micropipettes must be made on a microforge. These instruments are mounted in a micromanipulator so designed that the movements of the hand are transmitted as microscopic movements of the instrument in relation to the cell in the field of the microscope. By this method the physical properties of the visible components of the cell have been studied and qualitative chemical studies of limited character have been made.

It may be mentioned here that with the development of the interference microscope and the color translating microscope new physical methods have been provided for making certain qualitative and quantitative chemical analyses on fresh and living cells. The principles on which the use of these instruments is based are too technical and complex for us to discuss here.

The second method for the study of cell components has yielded more quantitative information. Large quantities of cells, obtained in one of the three ways already mentioned, are broken up in a cell homogenizer. The fragments are then suspended in an indifferent medium. The suspension is then subjected to centrifugation at different speeds. For example: The suspension of broken cells is poured into centrifuge tubes which are spun in the centrifuge at relatively low speeds for a short time. With this amount of gravitational force the intact cells and larger fragments will be packed in the bottom of the tube while the smaller components of the broken cells remain suspended in the supernatant fluid. The latter is carefully poured off into other centrifuge tubes and the pellet of intact cells and large fragments is discarded.

The supernatant fluid containing the suspended cellular components is again centrifuged at a slightly higher speed and for a somewhat longer time. With this gravitational force the nuclei, which are the largest structural components of the cells, will be packed in the bottom of the tube while the smaller components remain in the supernatant fluid. Again the latter is poured off into other centrifuge tubes for further centrifugation.

A small sample of the pellet is examined under the microscope to determine whether the nuclei are contaminated with other cell fragments or components. If so, the pellets are resuspended in the indifferent medium and the suspension is recentrifuged at the correct speed and for the proper time. The pellets of pure nuclei can then be subjected to biochemical analysis.

The supernatant fluid containing the smaller cell components is again centrifuged at a higher speed and for a longer time. With this force the mitochondria will be packed at the bottom of the tube while

the ultramicroscopic particles (or microsomes) remain in the supernatant fluid. Again the supernatant fluid is carefully poured off into other centrifuge tubes and the pellet is examined microscopically to determine the purity of the mitochondrial fraction. The pellets of pure mitochondria can be subjected to biochemical analysis.

The procedure is repeated again by centrifuging the supernatant fluid at the highest centrifugal speed for a long time to force the ultramicroscopic particles to the bottom of the tube. The final supernatant fluid containing the soluble proteins and electrolytes of the cell is collected and analyzed by biochemical and micro-analytical chemical methods. The purity of the pellet can be determined only with the electron microscope, as microsomes (or the submicroscopic particles) are too minute to be resolved by the light microscope. (Figure 3 & 4)[2]

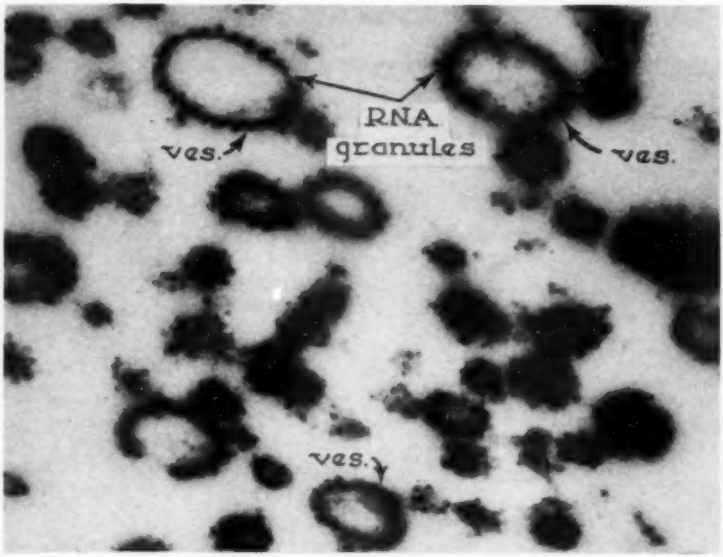

Figure 3[2]

The pellet of pure cytoplasmic particulates is subjected to biochemical and microchemical analysis. In this way the chemical composition of each structural component of the cell as well as the soluble substances in the cell juice can be determined and the chemical organization of the cell can be deduced.

It must be obvious that the quantities of some chemical substances localized in the various components of the cells or present at different

[2] Ham, A. W., 1957. Histology, 3rd Edn., p. 88, Fig. 65, and p. 89, Fig. 66. J. B. Lippincott Company, Philadelphia.

times in the functional states of the cell are too minute to be detected or measured by ordinary biochemical methods. For this reason micro-analytical methods, whereby minute amounts of material may be handled and analyzed, have been developed. These fall into three categories: 1) micro-volumetric (or gasometric) analysis 2) spectrophotometry and 3) paper chromatography.

The principle of chromatographic analysis is the differential rates of travel of the components of a mixture down an adsorbing column. The name chromatography originated because it was first used to separate colored compounds; but the method has been greatly extended so that many classes of colorless compounds can also be handled. Paper chromatography is a very ingenious method for the separation, identification and measurement of very small quantities of biochemically important compounds, such as sugars, amino acids, purines etc. which was developed by Consden, Gordon and Martin.[4]

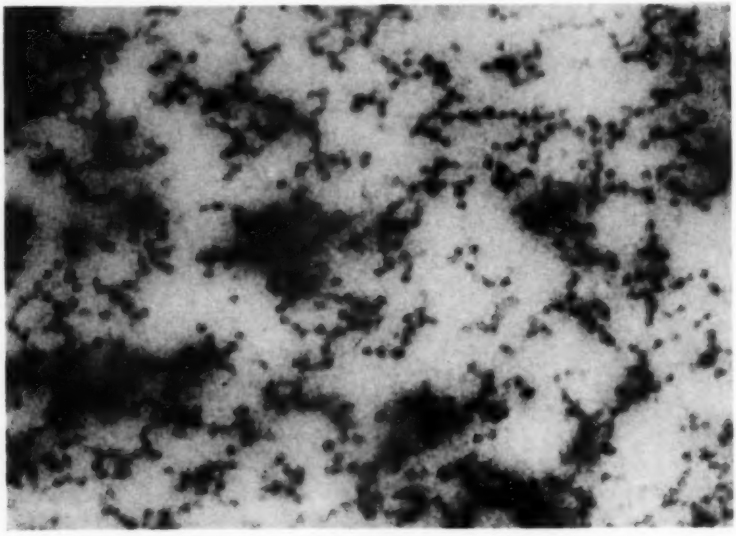

Figure 4[3]

The method of paper chromatography can be illustrated by a study of a mixture of amino acids in a tissue extract. A small measured quantity of the mixture in solution is placed as a spot near the top of a strip of filter paper which has been treated to remove all impurities from the cellulose. The paper is hung vertically from a trough con-

[3] Ham. A. W., 1957, Histology. 3rd Edn., p. 88, Fig. 65, and p. 89, Fig. 66. J. B. Lippincott Company, Philadelphia.

[4] Biochem. Jour. 1944, Vol. 38, p. 244.

taining a special solvent in such a way that the edge is immersed in the solvent. (Figs. 5A & B).

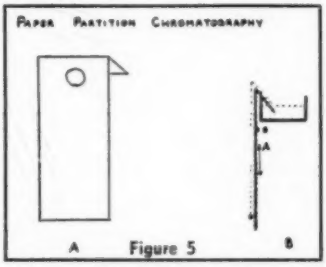

Figure 5

The solvent moves by capillary attraction down the paper and as it does so, some water (and solvent) becomes bound to the cellulose. Thus there is a moving liquid phase (A) flowing over the stationary cellulose-solvent complex (B). Each amino acid originally spotted on the paper will pass partly into the moving phase and remain partly in the stationary phase. If an amino acid has a higher affinity for the stationary phase it will travel slowly down the paper, but if it has a high affinity for the moving phase then it will travel rapidly. The various amino acids will, therefore, travel down the paper at different and characteristic rates so that when the irrigation process is stopped, the paper dried and sprayed with an amino acid reagent such as ninhydrin, we can see from the position of the different spots which of the amino acids was present in the original mixture spotted on the paper. (Figure 6: picture of chromatogram)[5]

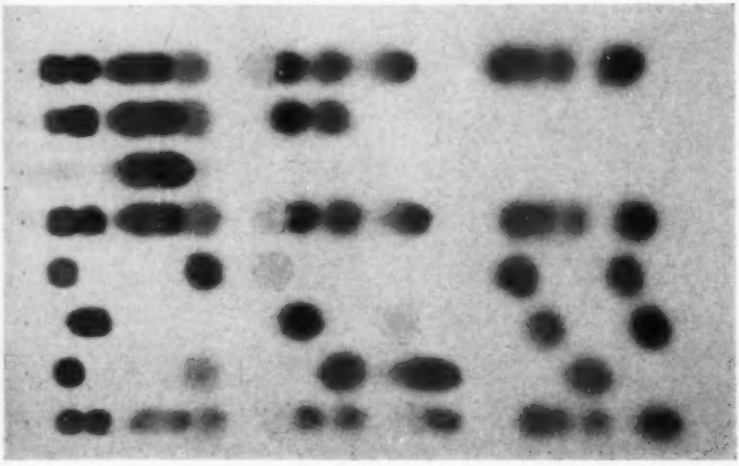

Figure 6[5]

[5] Courtesy of Professor C. S. Hanes, Department of Biochemistry, University of Toronto.

Individual amino acids are identified by comparing the position of the colored spots with standard chromatograms of known amino acids. Each colored spot on the test chromatogram can then be cut out and analyzed quantitatively by colorimetric methods.

Thus from a small quantity of a tissue extract or cell fraction the amino acids can be separated, identified, and measured quantitatively. By varying the solvent and the reagent, the sugars or the organic acids or the nucleotides in a mixture can also be analyzed in the same way.[6]

"In our enthusiasm for these methods, however, we must not forget that by far the most sensitive instrument for microchemical analysis is the living organism itself. The methods of immunology, for example, suffice to discriminate between compounds so closely related that the chemist is at a loss to distinguish between them. The genes revealed by genetic experiment exceed by an infinite multiple the meager number of nucleoproteins revealed by biochemical research. The bioassay methods depend on the exquisite sensitivity of the living organism to minute changes in its chemical environment. These are also microchemical methods."[7]

Now, most of the methods which have been discussed so far apply to the study of fresh tissues and cells. They have certain limitations and pitfalls, some of which should be mentioned. First of all, fresh tissue is chemically changing or changed tissue. Any manipulation or dissection of tissue destroys the relationships of its parts. Moreover with the physical dissection of tissues or cells some chemical substances may be lost and others may become adsorbed to the surfaces of other components, and other unknown chemical reactions and combinations may occur. Thus the precise identity and localization of chemical substances as they occur in the intact and living inidvidual cells or tissues is in doubt.

IV. Microscopic Histochemistry

Microscopic histochemistry seeks to overcome some of these limitations. This branch of Histochemistry is based on the production of microscopically visible microchemical reactions when chemical and physical tests are applied to tissue sections prepared for microscopic (or histological) study. It is almost as old as Biochemistry and Histology but has reached its fullest development only within the last ten years. This is made evident by the fact that nine textbooks and four journals devoted to microscopic histochemistry have appeared within the past nine years. (See list of references). And indeed the term Histochemistry has come to mean microscopic histochemistry in the minds of many.

The objective of microscopic histochemistry is to demonstrate, identify, localize, and measure chemical substances in their unaltered relationships within individual cells and tissues undisturbed by physical manipulations, under normal (or functional) and abnormal (or pathological) conditions. This requires a variety of technical procedures.

[6] Chromatography, 1954. Brit. Med. Bulletin, Vol. 10, No. 3. Ed. C. E. Dent.
[7] Bensley, R. R. 1949. Foreword to Glick's Techniques of Histo- and Cyto-chemistry. (See list of references).

The first requirement of microscopic histochemistry is to remove a small portion of the tissue from the body with as little damage as possible and to so treat it as to preserve it against any further chemical, physical or structural change. The latter has been attempted in a number of ways: by instantly freezing solid the tissue and then removing the frozen water from the frozen tissue in vacuo at very low temperatures, by boiling the tissue, by immersing the tissue in a chemical preservative (or fixative).

Investigators have always sought for a preserving fluid which would maintain the elements without loss or distortion of the structures originally present in the living cell and without addition of artificial structures and substances (or artefacts) produced by precipitation of substances present in solution in the living tissues. This goal has never been reached because, however unobjectionable a preserving fluid may be, nevertheless, its first duty is to render the substances in the cell insoluble, and this phenomenon involves precipitation. One must, therefore, try to select a preserving agent which precipitates in the ultramicroscopic range, so that the precipitation phenomena intrude as little as possible into the picture of the preserved cell.

The agents which are least objectionable from this standpoint are: 1) osmic acid 2) formaldehyde and 3) bichromate of potassium. Useful but more objectionable agents are alcohol, acetone, acetic and picric acids and various combinations of these agents. The choice of the method of fixation is determined largely by the purpose and nature of the histochemical study.

The next requirement is that microscopically thin slices be cut from the preserved tissue. For this purpose the tissue must usually be embedded in a firm or rigid medium such as ice, wax, mitrocellulose, a plastic or resin. The thin slices are obtained by the perfect edge of a special knife, blade, or plate glass mounted with the block of tissue in a precision instrument called a microtome. The microscopic sections are then usually mounted on glass slides in such a way that they adhere to the glass slide. The sections are then ready for the application of the physical or chemical tests. They may be examined immediately under the microscope or they may be covered with an optically homogeneous material and thin glass cover slips to become permanent preparations. The results of the tests are interpreted, evaluated and measured or recorded photographically through the microscope.

The histochemical tests fall into three main categories: physical, chemical and pseudochemical. The physical methods include micro-incineration[8], microradiography[8], emission spectrography[8], ultraviolet absorption microspectrophotometry[9], X-ray diffraction[9] and autoradiography—about which you will hear more in Dr. Norris' paper.

The chemical and pseudochemical methods constitute by far the greatest number of tests that are commonly used. For the chemical reactions that are involved and the details of technique a good text of Histochemistry should be consulted. Although each text has its own

[8] Medical Physics, ed. Otto Glasser, Chicago, Year Book Pub. Inc. 1944, pp. 729-733, 1446-1457.
[9] De Robertis, E. D. P. Nowinski, W. W. and Saez, F. A., 1954. General Cytology 2nd edn. Philadelphia, Saunders Co., pp. 195-197, 93-95.

merits, the two which I have found most practical are Pearse's Histochemistry (1953) and Casselman's Histochemical Technique (1958). (See list of references). The chemical tests are based on the production of a microscopically visible substance due to a chemical reaction. The pseudochemical methods depend on the staining of substances and structures by selective dyes which are so consistently specific as to identify the substance or structure.

Six classes of substances have been demonstrated and identified by histochemical tests applied to tissue sections: carbohydrates, lipids, nucleic acids, proteins, certain inorganic constituents, and vitamin C. The carbohydrates can be subdivided into 1) simple sugars 2) simple polysaccharides and 3) mucoid substances. The simple sugars (with possibly one or two exceptions) are too soluble to be detected in tissue sections. But the polysaccharides, such as glycogen, and the mucoid substances, such as acid mucopolysaccharides, and glycoproteins can be precipitated in the tissues by suitable fixing agents such as alcohol.

One of the earliest histochemical tests was the iodine test for starch and this remains a useful test for glycogen, which is an animal starch. These complex carbohydrates can also be demonstrated in the following way. The tissue sections are treated with an oxidant, such as periodic acid which converts the sugar alcohols or glycols to aldehydes. The aldehyde can then be detected by a specific reagent called Schiff's reagent. This reagent is rosaniline hydrochlorate (basic fuchsin) decolorized by sulfurous acid. When it combines with the aldehyde, a violet to red color is produced. (Although this reaction is credited to Schiff it was first reported fifteen years earlier by Lauth, the discoverer of thionin, who used it in controlling the fermentation of beer for the detection of aldehydes as an impurity in the alcohol.) It has been found that only the complex sugars containing a glycol in the alpha position give this color reaction with Schiff's reagent. Therefore complex sugars with a slightly different molecular structure cannot be detected by this method.

Complex carbohydrates can also be demonstrated by selective stains. These include Best's carmine, mucicarmine, muchematein, and metachromatic staining as with toluidine blue. Alcian blue is thought to be specific for acid mucopolysaccharides and these mucoid substances can also be demonstrated by color reactions for iron or copper after tissue sections have been exposed to and have selectively adsorbed these minerals in solution.

The polysaccharides can also be distinguished from the mucoid substances by the use of specific enzymes. For example, if two similar sections of the same tissue are used and one is treated with a starch-splitting enzyme so that the polysaccharides are broken down to soluble sugars and washed away and then both sections are stained by the P.A.S. method, those areas which are Schiff-positive in the untreated section and Schiff-negative in the section treated with starch-splitting enzyme will indicate the distribution of polysaccharide, whereas those areas which are Schiff-positive in both sections will indicate the distribution of the mucoid substances. In the same way the differentiation of the acid mucopolysaccharides is analyzed by the use of specific enzymes.

The distribution of lipids can be demonstrated in two ways: 1) by fixation of the tissue in osmic acid, and 2) by the use of fat soluble dyes. Since lipids are soluble in reagents that are commonly used in preparing tissues (fixed in agents other than osmic acid) for embedding them in a firm medium, most of the tests for lipids must be made on frozen sections (that is, sections cut from a frozen block of fixed tissue).

There are many classes of lipids which can be distinguished by differential and specific tests. Acidic and non-acidic lipids can be differentiated with nile blue. Unsaturated lipids are identified by the Schiff reagent after oxidation with performic acid. Compound lipids can be differentiated by selective extraction methods.

Phosopholipids can be identified by the use of four tests: sudanophilia (especially Sudan black B), the reaction with nile blue, the acid haematein tests and by selective extraction with acetone and alcohol. Identification of glycolipids is made by the use of three tests: sudanophilia, the PAS test and by selective extraction. Cholesterol and its esters are demonstrated by digitonin, the Lieberman reaction, or by the Lifschutz reaction. The simple lipids are identified by exclusion, that is, those lipids which are colored by fat-soluble dyes, but do not react to other specific tests are considered to be simple lipids.

There are several general methods for detecting proteins in tissue sections but the most differential methods are 1) selective extraction by means of enzymic hydrolysis with allegedly specific proteolytic enzymes and 2) fluorescent antibodies. The latter method is one of the most recent advances in histochemistry. It depends on the facts that 1) proteins and protein-like materials are antigens which provoke an antibody reaction in the body, and that 2) specific antigens combine selectively with their specific antibodies. Thus, for any given protein an antibody can be produced in the blood serum of another animal. The antibody is then isolated and combined with a fluorescent dye. When a solution containing the fluorescent antibody is applied to a tissue section the antibody combines only with the specific protein in the section. The section, after it is washed, is examined in ultra-violet light. The distribution of the specific protein in the section is detected by the fluorescence of the protein-fluorescent-antibody complex.

Some of the classical biochemical methods for the identification of various amino acids have been modified for use on tissue sections. These reactions determine only a fraction of the constituent amino acids of any particular protein, but a positive reaction is indicative of the presence of a protein, since free amino acids rarely occur in microscopically detectable quantities in normal tissue.

For histochemical purposes the proteins can be divided into 1) simple proteins, defined by the biochemists as yielding on hydrolysis mainly a-amino acids and their derivatives and 2) conjugated proteins, which yield substantial quantities of non-protein substances in addition.

The four most important types of proteins in tissue sections are nucleoproteins, enzymes, some hormones and fibrous or structure proteins. These are widely distributed and occur both within cells and in intercellular substances.

Table 2

Simple Proteins	Conjugated Proteins
Albumins	Nucleoproteins
Globulins	Mucoproteins
Albuminoids	Glycoproteins
(Scleroproteins)	Lipoproteins
(Structure proteins)	
Globins	
Histones	

In general, the proteins and protein-like materials tend to adsorb basic dyes such as azure, toluidine blue, and pinacyanol which impart a blue color to the tissue sections, but in various combinations and varying degrees of acidity some, at least, may adsorb acid dyes such as eosin, erythrosine and acid fuchsin. The nucleoproteins are combinations of a basic protein with various nucleic acids derived from phosphoric acid, purine or pyrimidine bases and pentose or desoxypentose sugar. The nucleic acids are said to be basophilic because those areas in the tissue where they occur stain intensely with basic dyes. These have been detected and measured by means of ultra violet absorption microspectrophotometry. Two types of nucleic acid have been identified in tissues. pentose—or ribonucleic acid (RNA) and desoxypentose—or desoxyribonucleic acid (DNA).

These two nucleic acids can be distinguished in tissue sections in three ways: 1) by the elective absorption of the dyes pyronin and methyl green, 2) by selective extraction by means of specific enzymes and 3) by the Feulgen reaction. On mild hydrolysis with dilute hydrochloric acid, aldehyde groups are released from the desoxy pentose sugar but not from the pentose sugar. The aldehydes so liberated can combine with Schiff's reagent to produce a purple-red color. Thus, the DNA is Feulgen positive whereas the RNA is Feulgen negative.

Microscopic histochemical tests have been developed for only a few of the enzymes. These tests, in general, involve the application of the specific substrate, for the particular enzyme to be tested, to the tissue section at the right acidity. The enzyme reacts with the substrate presumably only at those sites in the tissue section at which it occurs, releasing substances from the substrate which are either visible microscopically because of their color or can be precipitated as visible compounds by excess metallic ions added to the substrate. These visible reaction products presumably indicate the localization and quantity of the enzyme present in the tissue section.

Of course one major requirement for this type of test is that the tissue must be so prepared as to immobilize the enzyme and at the same time retain its chemical activity.

A few of the protein hormones have been identified and localized in tissue sections by means of the specific fluorescent antibody method. And a few of the fibrous or structure proteins have been demonstrated by selective extraction using apparently specific (hydrolysing or depolymerizing) proteolytic enzymes.

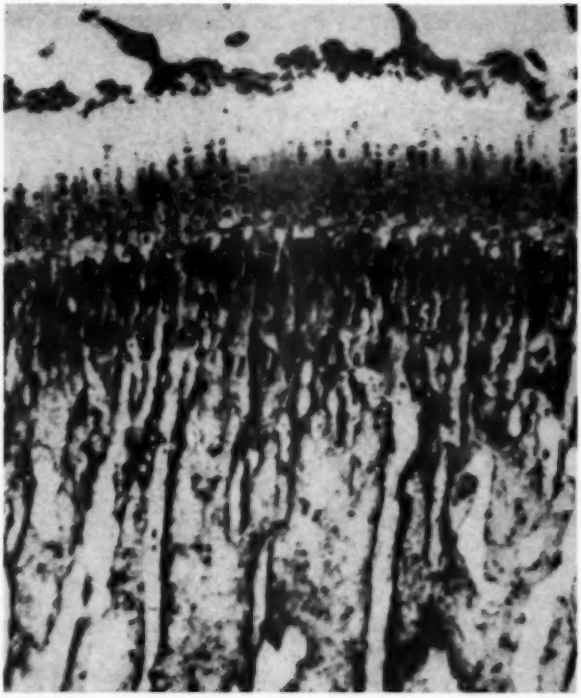

Figure 7
Test for alkaline phosphatase in section of growing end of long bone.[2]

Finally, some of the inorganic constituents of tissues have been demonstrated histochemically. Most of these constituents are too soluble to be accurately localized by microchemical reactions and are better demonstrated by microincineration, emission electron microscopy, or emission microspectroscopy. However, microchemical reactions are commonly used for demonstrating the insoluble forms of calcium and iron. These are of two main types 1) metal or electrolyte substitution and 2) the production of dye-lakes which then color the sites of calcium or iron localization. Thus calcium can be demonstrated by its substitution with silver as with the method of von Kossa (Figure 8). Similarly iron can be demonstrated by substituting sulphide to give iron sulphide which possesses a dark, greenish black color, or ferrocyanide which gives the

[2] Ham, A. W., 1957. Histology, 3rd. Edn. p. 284, Fig. 189. J. B. Lippincott Co., Philadelphia.
[3] Ham, A. W., 1957. Histology, 3rd. Edn. p. 285, Fig. 190. J. B. Lippincott Co., Philadelphia.

Prussian blue reaction. Iron can also be demonstrated by forming a lake with hematoxylin.

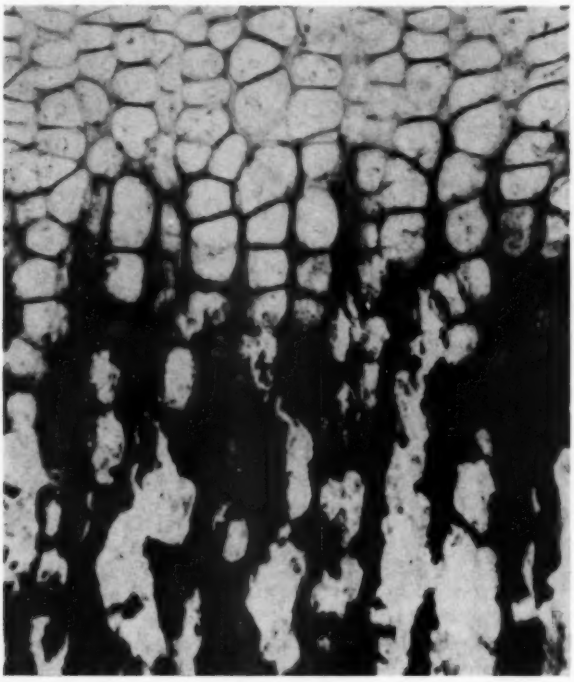

Figure 8
von Kossa method for calcium in a section of the growing end of a long bone[3] or by forming a lake with alizarin or hematoxylin.

V. Limitations and Pitfalls of Microscopic Histochemistry

As we have seen, the purpose of microscopic histochemistry is threefold: namely, recognition, localization, and quantitation of specific substances in cells and tissues. The first of these may be accomplished equally well or better by macrochemical (or biochemical) methods and the third is achieving importance through the newer physical methods of measurement. Accordingly, localization becomes the chief function of microscopic histochemistry. In order to serve this purpose a microscopic histochemical test must fulfill the following conditions:

1) It must be an inevitable reaction between substances of known chemical nature.

[3] Ham, A. W., 1957. Histology 3rd Edn., p. 285, Fig. 190. J. B. Lippincott Co., Philadelphia.

2) It must yield a reaction product which by reason of the formation of crystalline or amorphous precipitates or by the production of color is visible under the microscope.

3) The reaction must occur at the site of the substance to be tested and the reaction product must remain where it is formed and not be leached out or migrate to another site.

In animal tissues all of these conditions are seldom realized. There are, therefore, three main limitations in microscopic histochemistry:

1) The tests so far devised, in general, serve to identify certain classes of substances, rather than the precise chemical composition of any substance.

2) The tests are, at present, far from standardized. Many unknown or undetermined factors exist which may interfere with the successful application of many of the tests in the hands of different investigators.

3) The interpretation of the results of microchemical tests applied to tissue sections is fraught with pitfalls. Some of these should be mentioned.

The reactions which occur in the complex colloidal matrix of cells and intercellular substances are quite different from those of more simplified systems in the test-tube. Fixing agents (such as formaldehyde and alcohol) may form additive compounds which give quite different reactions from the substances they are used to preserve. Ion mobility may be so great that color reactions take place outside of the tissue and the dispersed reaction product is then adsorbed to suitable surfaces due to surface charges. Or, the reaction product, though produced in the tissue may be so highly dispersed that it drifts to other locations due to surface charges. Or, the rate of formation of an insoluble product may be so extended by reason of the protective action of the protein matrix that the product of the reaction may move from the site of original formation to another site. Where mere traces of substances are detected by the formation of highly colored products, the possibility that they have been derived from glassware, instruments or reagents and been merely adsorbed by the section, much as a stain is adsorbed, must be constantly kept in mind.

Microchemical identification which depends on selective extraction is open to skepticism. The specific enzyme or extractant may be merely masking or interfering with the chemical or physical stain rather than removing the substance from the section. Likewise, the results of microchemical tests based on substitution methods should be accepted only with reservations and the possibility of the adsorption of the substituting ions, independent of the reactions supposed to occur, should be entertained.

Negative results are worthless. The limitations of histological technique, the many factors which interfere with chemical reactions, and the very thinness of the preparations make any negative conclusions invalid.

These limitations of microscopic histochemistry are being gradually reduced as chemists become more aware of the physical and chemical complexities of cells and tissues and microscopists become better chemists and both seek to determine the unknown factors which interfere with micro-chemical reactions in tissue sections and devise new and

better and more precise methods.[10]

VI. The Value of Microscopic Histochemistry

Imperfect as it is, nevertheless microscopic histochemistry provides some of the most valuable methods for the chemical analysis of cells and tissues. It has provided new techniques for demonstrating structures and changes in structure which classical staining methods fail to do. It provides suggestive evidence for the presence in tissues of certain classes of substances many of which can then be confirmed by combining the techniques of microdissection and micro-analytical chemistry. It is the only method we have for localizing substances within the *individual* cells of an intact organ or tissue.

VII. Summary and Conclusions

Thus we can see that histochemistry holds a key position in research in Cytology and Histology by revealing more completely the structure and the nature of structure of cells and intercellular substances. It extends research in Biochemistry to the analysis of chemical reactions within the various units of living matter. And by determining the normal and abnormal chemical reactions which occur in all the various types of cells of organs and tissues it provides the basis for cellular Physiology and Pathology.

Although each of the branches of Histochemistry-microdissection, micro-analytical chemistry and microscopic histochemistry,—has its limitations and yields results that are only approximations, the judicious and skillful combination of all these methods can give us a close approximation of the chemical structure and the chemical reactions of living cells and tissues.

The future of Histochemistry is bright with promise. Mindful of the pitfalls, new investigators will continue to uncover and resolve complicating factors, standardize techniques, and devise new techniques for identifying, localizing and measuring all the chemical substances and reactions in all types of tissue and thus provide the chemical and physical basis of life itself.

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A FAMILIAL STUDY IN PORPHYRIAS AND PORPHYRINURIAS*

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Abnormalities in pigment metabolism are thought to be the result of inborn errors of metabolism. These disorders involving pigments may include phenylpyruvic oligophrenia and ochronosis to mention only two. The presence of porphyrins in excessive amounts can be classed along with these abnormalities.[2] Because almost all systems of the body are affected in these disorders, porphyrias and porphyrinurias are of diagnostic interest.

In this paper the different systems of the body involved as well as the aspects of inheritance will be demonstrated through data collected in a comprehensive study of two families.

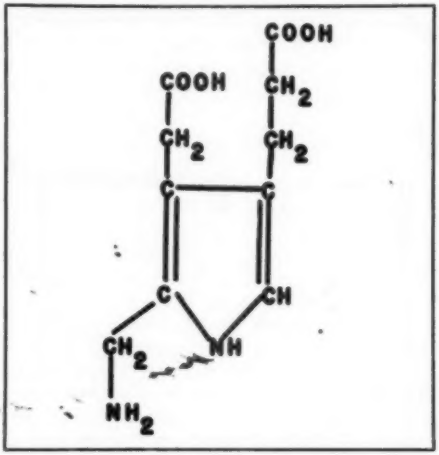


Figure 1
The structural formula of porphobilinogen, a precursor of the porphyrins.[3]

Some authorities seem to think that porphyria is inherited as a dominant gene,[5] while others regard it as a recessive gene.[9] From results of the studies to be presented here one can assume this characteristic to be dominant.

The porphyrin compound is widely distributed in nature. For example, it is contained in hemoglobin and chlorophyll.[1] Prophobilinogen, a simple organic structure[3] containing one pyrrol ring, is a precursor of the porphyrins and is important in their metabolism (see Figure 1). Arthur Grollman states that, "Porphyrins are red or purple pigments which, as an iron-porphyrin complex, constitute the prosthetic group of hemoglobin and cytochrome. The porphin nucleus consists of four pyrrol rings joined by methene bridges."[2] The radicals essential to completion

* Read before the 26th Anniversary Convention of ASMT, Milwaukee, Wisconsin, June, 1958.

of the porphyrin molecule are one or a combination of the following: Methyl group (CH), vinyl group (CH = CH), propionic group (CH — CHOOH). Any of the above three radicals may be joined at one of the numbered points in the structural formula of the porphin in nucleus (see Figure 2).

Due to the nature of the structure of the porphin nucleus the number of probable isomers is greatly enhanced. When the same radical group is present on all four pyrrol rings, four stereoisomers are possible. These are designated as Types I (1, 3, 5, 7), II (1, 4, 5, 8), III (1, 3, 5, 8), and IV (2, 3, 5, 8), where the indicated position supports a methyl group and an alternative radical occupies the remaining sites. However, only Types I and III occur in nature.[2]

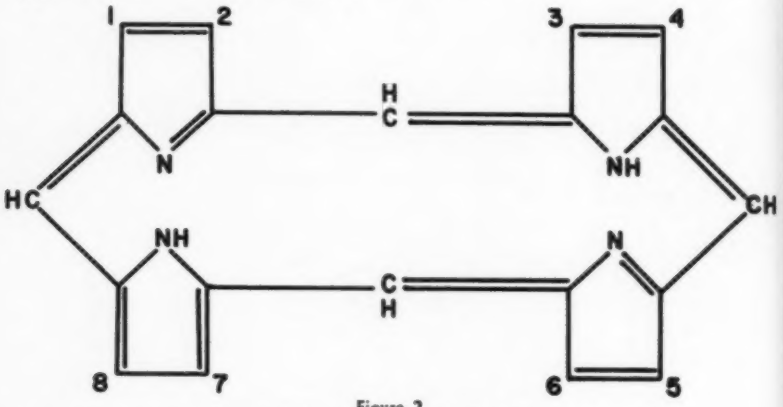


Figure 2
Structural formula of the porphin nucleus.

Grollman further states that: "The porphyrins are amphoteric substances which are extracted by organic solvents from neutral for faintly acid solution. They may be estimated by their intense red fluorescence in ultraviolet light and identified by their chemical behavior, their adsorption spectra, their chromatographic properties and the melting points of their methyl esters."[2]

Table I
WAVE LENGTHS TO DETECT AND DIVIDE THE DIFFERENT PORPHYRINS[3]

Type of Porphyrin in 25% HCl	Wavelength maxima, in mu
Protoporphyrin	557.2, 582.2, 602.4
Hematoporphyrin	508.7, 524.8, 548.7, 572.4, 593.1
Deuteroporphyrin	549.3, 572.6, 592.4
Uroporphyrin I, III	511.3, 526.6, 553.6, 577.6, 597.9
Coproporphyrin I, III	550.9, 574.6, 593.9
Mesoporphyrin	508.7, 524.8, 548.7, 572.4, 593.1

There are many nomenclatures for porphyrin disorders but an eclectic adaptation of these will be used here. The two main divisions of the classification are hereditary and acquired. Further subdivisions are: I, Photosensitivity; II, Abdominal manifestations; III, Neurological; IV, A combination of any of the previous three. Congenital, chronic, acute, or a combination of the last three conditions may manifest themselves in any of these subdivisions.

One system classifies the disease in this manner: 1. acute, with episodes of abdominal and/or nervous manifestations, 2. chronic, with late appearance of photosensitivity with or without abdominal and nervous symptoms and, 3. congenital, with early photosensitivity.

Positive findings may be obtained from some patients with "1. neoplastic disease, 2. severe liver disease, and 3. nervous system disease."[4] Also, the administration of certain drugs such as the sulfonals, trinols, and the barbiturates will give positive results for porphyrins.[6] The basis for positive findings has not been explained.

Procedure

The procedures used in this study were in the following sequence: 1. urine fluorescence test for porphyrin, 2. a family history and case histories, 3. a second urine fluorescence test, 4. a urine test for porphobilinogen, 5. a complete blood count, noting particularly such things as basophilic stippling or polychromatophilia, 6. reticulocyte count, 7. battery of liver tests, (e.g. B.S.P., alkaline phosphatase, thymol turbidity, total and direct bilirubin, etc.) 8. blood fluorescence test, and 9. bone marrow fluorescence test for porphyrins.

The protocols used in the various tests were as follows:

1. Fluorescence Test for Porphyrins in Urine (as determined at the University Hospital).

Reagents: Porphyrin mixture composed of 1 part ether, 1 part glacial acetic acid, and 3 parts 95% ethyl acetate.
Sodium hydroxide—5% aqueous solution
Hydrochloric acid—50% aqueous solution

Procedure:
1. Extract approximately 30 cc. of urine with about 20 cc. porphyrin mixture in separatory funnel, saving the lighter mixture layer found on top.
2. Wash with about 30 cc. distilled water and pour off washings in bottom layer.
3. Wash with about 30 cc. NaOH and pour off bottom layer of washings.
4. Wash with approximately 30 cc. distilled water again and pour off washings.
5. Wash with about 30 cc. HCl and save washings.

1. The washings from step 5 and a blank of distilled water should be placed in non-pyrex tubes (pyrex has a fluorescence of its own) and examined immediately in a completely dark room against a black background with an ultra-violet lamp. If porphyrins are present a red fluorescence will be visibly present. Ether may be added to the mixture when washing with water in steps 2 and 4 to give a sharper junction. Any vitamins present which might interfere with the test are removed in step 3. It is not advisable to use rubber stoppers at any point during this procedure, because rubber gives a blue fluorescence which might interfere with the color perception.

2. When securing a case history on the patient the attending physician should be requested to obtain a partial family history, noting in particular if other family members present symptoms which might indicate porphyria or porphyrinuria. It is also important to obtain a drug history. Among the many possible symptons of these metabolic defects the most common are: abdominal colics; nervous disturbances which

may be manifested as localized paresis, psychotic states, convulsions or sensory disturbances; and severe cutaneous symptons due to photosensitivity. Any of these may appear early or late in life, depending mainly on whether the disease is congenital, chronic, or acute.

3. Watson's test, a widely published procedure requiring no elaboration, was the method of determining porphobilinogen used in this study.[7] Porphobilinogen if found during an attack of porphyria and porphyrinuria is of diagnostic value.

4. Next, a complete blood count, with particular emphasis being placed on the presence or absence of basophilic stippling and the appearance of any marked polychromatophilia is done. Basophilic stippling might suggest lead poisoning rather than the suspected porphyrin manifestations, whereas marked polychromatophilia usually is one criterion of porphyria.

5. A reticulocyte count is the next procedure to be followed. In a true porphyria the count is usually increased, particularly if polychromatophilia is absent in the differential count. Any standard procedure for obtaining the number of reticulocytes is permissible.

6. Complete liver studies should be done on the suspected patient. Usually all results of the battery of liver tests will fall within the normal range with the exception of the BSP, which shows an elevated retention. The procedures for these tests may be any of those routinely carried out by a clinical chemistry laboratory.

7. The last two steps of this study were blood and bone marrow fluorescence tests for porphyrin. These procedures should be carried out by the same person, preferably one who is familiar with fluorescence tests. The technique[3, 4] is to set up a microscope with an ultra-violet lamp attachment in a completely dark room and then examine wet, fresh preparations immediately after obtaining the blood and bone marrow. If porphyrins are present the affected cells will emit a red fluorescence. Nucleated red cells in the bone marrow are prominent under ultraviolet treatment. Occasionally an increase in normoblasts will appear in the total bone marrow differential.

Familial Study

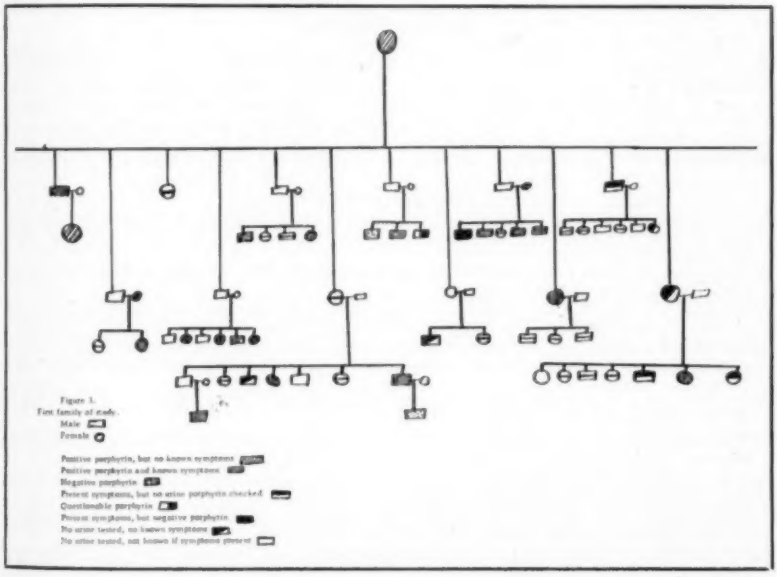
The first family study was done in November, 1956, on a white family of the Mississippi Delta region. Because of the large number of people involved and because we were unable to bring these subjects back for further testing, only the first two procedures were completed. Histories were obtained from forty-five members of the maternal side of this family and urines obtained on forty-three. Forty positive urine porphyrins were demonstrated by the fluorescence test, giving 92% positive and 8% negative results. Of the members in this family with positive findings, 52.5% were females, and 47.5% males. All the negative results were on males. The age ranges represented were: Females, positive, 2-37 years; males, positive, ½-31 years; negative 3-37 years.

Twenty-one, or 48.8% of the forty individuals with positive urine porphyrins had various manifestations of porphyria or porphyrinuria. Four showed evidence of abdominal manifestations, with one of this number having had an appendectomy and one receiving treatment for appendicitis without surgery. Early photosensitivity appeared in one

patient, dark urines in two, discolored teeth in one, severe heat rash in three, summer blistering in two, and a rash which became worse on exposure to the sun in three. There were neurological symptoms and a retarded bone age in one infant, and a bone age at the lower limits of normal in another. One person who had a negative porphyrin at the time of study had a history of abdominal pains followed by an appendectomy. Two members of the family absent at the time of the study were reported to have abdominal symptoms and summer blistering.

Control urines were run on employees and staff of the Medical Center. Of eleven control determinations one was positive. This was surprising in view of the fact that none of the control subjects had known histories of porphyrinuric symptoms. Upon investigation it was found that the positive urine in the control series was from a person who had received electrical shock treatment, suggesting psychotic or neurological symptoms of the disease. In this series controls showed 91% negative and 9% positive results. Controls were run on several natives of the same Delta region with negative results. Controls run on a person who had visited this area were also negative. Thus constitutional rather than environmental conditions appeared to be paramount.

The second study was made on the family of a white, 46 year old male from the central area of Mississippi, beginning November, 1957. Complete studies were possible because the principal subject was hospitalized with a porphyrinuric attack at the time of study. He had a previous history of abdominal surgery without relief of symptoms. Tests for urine porphyrin fluorescence and porphobilinogen at this time were



positive. A battery of liver function tests were normal except for an increased BSP retension of 24%. The blood and bone marrow fluorescence tests were positive. The reticulocyte count was increased to 3.2%. His complete blood count was essentially normal except for a white count of 20,700 per cubic millimeter. This patient was unable to walk although he could move his legs. He showed no photosensitivity and had no previous history of photosensitivity.

Urines were obtained from three other members of this family: A daughter and two sisters. One sister, a registered nurse, reported a history of abdominal pains which persisted even after an appendectomy. Positive urine porphyrins were also found in these three members of the family.

In conclusion, it appears evident that hereditary influences play a major role in porphyria and porphyrinuria. It has been proven that both male and female, ranging in age from infancy to adulthood, are affected. There is a slightly higher percentage of females with the disease. A complete history of the patient and a partial history of his family is essential to make a final diagnosis of these disorders.

Summary

Some of the many systems of the body involved in porphyria and porphyrinuria have been presented as well as the hereditary aspects of these metabolic disturbances. After giving a basic summary of the chemistry of porphyrins, the techniques used for detection of these were outlined. Diagnostic findings of the disorders were presented through a familial study, with pertinent data from two family groups.

Acknowledgment

The author acknowledges with sincere gratitude the inspiring interest and assistance of Margaret B. Batson, M. D., Acting Assistant Professor; Department of Pediatrics; and Warren N. Bell, M. D., Director of Laboratories & Associate Professor of Medicine; Department of Medicine; University of Mississippi Medical Center. Also to Rita Krestensen, M.T. (A.S.C.P.); Department of Pathology; University of Mississippi Medical Center, and Joseph Blackman, M.T. (A.S.C.P.); Department of Laboratories, Mississippi Baptist Hospital, Jackson, Mississippi.

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THE ERYTHROCYTE SEDIMENTATION TEST—AN HISTORICAL REVIEW*

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It may seem strange to you that I have selected as my topic a review of the history of such a simple every day test as the determination of the erythrocyte sedimentation rate. I hope, however, that after you have heard the story you will agree with me that sometimes a commonplace thing may have an undiscovered fascination.

We will begin our story with Robin Fahraeus, a Swedish pathologist who, as an intern, re-discovered a phenomenon of practical significance, which though it had been the cornerstone of ancient medicine, had been discarded as nonsensical folklore when Virchow and others made their great discoveries and formulated their theories which so clearly explained the cause of much of disease and laid the foundation for pathology as we know it today. When, in the 1850's, Virchow with the help of the microtome, the microscope and staining methods discovered with certainty that disease was related to cellular changes and cellular reactions and that there was a characteristic destruction and re-organization of the cells in tuberculosis, syphilis, cancer, pneumonia and other diseases, an entirely new approach to medicine was introduced. We can safely say that much of the great discovery in medicine during the last one hundred years stems from his enthusiastic leadership in advancing these theories and concepts. As often happens, when answers to centuries of speculation and groping come suddenly into man's hands, he frequently dismisses what his ancestors relied upon so much and in his enthusiasm for the new soon forgets what seems to no longer have any practical value.

The observation that blood removed from persons who were ill frequently separated into layers which appeared in the blood clot was familiar to the ancient Greeks. In fact, this phenomenon became the basis for the medical verification of the ancient cosmology developed in its final form by Pythagorus in the 5th Century, B.C.

Before discussing the Pythagorean concept of matter, it will be necessary to describe the blood clots that physicians and others observed in those times. The examination of the blood clots by the ancient Greeks can be described as probably the first hematologic test recorded in history. The Greeks occasionally used amber vessels for containing liquids. Amber, like paraffin, many plastics and silicone has a water repellant surface. All of us are familiar with the effects which these surfaces have on retarding blood coagulation. If blood is collected in a test tube made of lucite or a similar plastic, the coagulation time may be delayed as much as three times the coagulation time in glass. If the erythrocyte sedimentation is even only moderately rapid, the erythrocytes will begin to settle many minutes prior to coagulation if the blood is allowed to stand without motion in a lucite tube. In our fancy, we can see an ancient Greek surgeon attempting to stanch the flow of blood from a battle wound. He may have had an amber vessel handy which collected the blood freely flowing from the large wound. Since many of the soldiers had chronic malaria, it was possible that they had a rapid sedimentation

* Presented at the 26th Annual Convention of ASMT, Milwaukee, Wisconsin, June, 1958.

rate. Other soldiers who were healthy had normal sedimentation rates. Sooner or later it would become obvious to those who looked at the clots in amber vessels that there were differences in their appearance. These crude observations were eventually correlated and studied by the scientists of that time and were interpreted in terms of Pythagorean cosmology. FIGURE 1 shows what they observed when the blood was removed from a healthy person. FIGURE 2 describes what they observed when the blood was removed from a person with a rapid sedimentation rate.

This phenomenon can be easily reproduced today if we collect fifty or more cc of blood and use a plastic vessel with a diameter of four or more cm.

The misinterpretation of this factual observation caused Western medicine to be dominated by a curious theory of disease for more than two thousand years.

The Pythagorean cosmology held that all matter was made up of four elements: fire, earth, air and water mingled together in combinations which accounted for all of the phenomena in nature. When this theory was applied to man, it became the humoral theory of disease and remained in modified forms as the explanation for human biological processes all through the Roman and Medieval periods and persisted in curious variations until the last century and still remains in isolated examples of folk medicine even today. The theory was summed up by Hippocrates in these words:

"The human body contains blood, phlegm, yellow bile and black bile, and it is these which constitute her bodily nature and which are the origin of sickness and health. The human being is healthiest when these elements with respect to combination, strength and quantity stand in well proportioned relationship to one another and are as much as possible intimately mixed: whereas, she suffers when any one of these matters is present in too small or too great a quantity or has separated and is not combined with all the rest."[1]

Let us again refer to FIGURE 2. The oxygenated erythrocyte zone was known as the true blood and was thought to be related to the air, the serum or yellow bile to fire, the fibrin cake or phlegm to water and the de-oxygenated erythrocytes or black bile to earth. These parts of the blood represented in their pure form the four humors which, when in proper balance signified good health. The four humors have indeed become a part of our literature and our common speech even today. The plays of Shakespeare contain a wealth of allusions to the humors, and even today we refer to them as descriptive of personality when we speak of sanguine, choleric, phlegmatic and melancholic people.

One can now readily understand how therapeutic venesection became the principal method of treating illness during the Middle Ages. If a person coughed up sputum as a result of tuberculosis or pneumonia, it was reasoned that the patient's humors were out of balance and he was trying to rid himself of the excess phlegm by expectoration. After all, did not the sputum look and feel like the fibrin cake of the blood? Certainly, the blood removed had a lot of phlegm. If the patient had dysentery, cholera or typhoid fever, he expelled copious yellowish watery feces. Obviously, he was trying to rid himself of yellow bile. Therefore, why

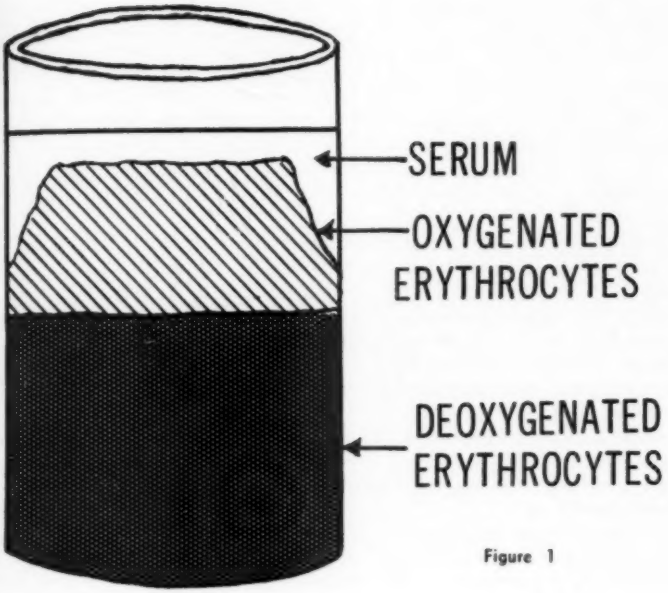

Figure 1

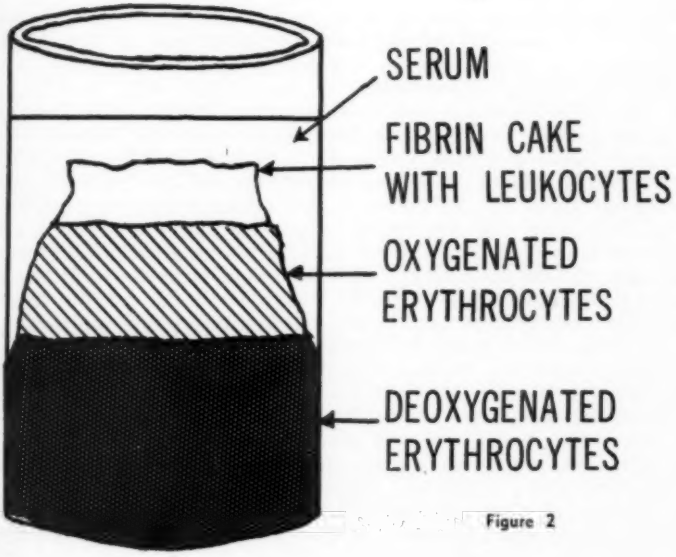

Figure 2

not assist him by venesection. The system became very elaborate and complicated and each variation in technique had a special purpose. Galen, the Roman physician, whose writings dominated the West for over a thousand years, described eight "dyscrasias" which could give rise to disease. Incidentally, this is the only etimologically proper use of the word "dyscrasia" (dys-crasis, Gr., bad mixing). An untold amount of differentiation and combination of the dyscrasias accounted for all illness. Thus, the phenomenon of erythrocyte sedimentation dominated the erroneous thinking of physicians more than any other scientific observation for centuries.

Fahraeus has quoted some Swedish maxims recorded in 1662. They serve as a good summary for this portion of our discussion.

"Man is fashioned from four elements, Earth, Water, Air and Fire, and takes his nature from that he has most of. From the earth which is cold and dry, he becomes melancholy, heavy and earthy, is likened to the nature of autumn and earth.

From water is man cold and humid, white colour, phlegmatic, likened to nature of water and winter.

From wind he is warm and red, beautiful, sanguinal, is likened to the nature of spring and air.

From fire he is hot and dry, modest, choleric, is likened to the nature of summer and fire."[1]

"When thou hast let blood.
So let the blood stand so long
Till it becomes cold and so shalt thou understand
That then thou canst see the nature of the blood
And what blood-letting have for power."[1]

It was John Hunter, who, in 1786, really made the basic discovery of the cause of erythrocyte sedimentation, namely rouleaux formation. "In all inflammatory dispositions in the solids, whether universal or local, the blood has an increased disposition to separate into its component parts, the red globules become less uniformly diffused, and their attraction to one another becomes stronger so that the blood when out of the vessels soon becomes cloudy or muddy and dusky in its colour, and when spread over any surface it appears mottled, the red blood attracting itself and forming spots of red,"[1] and it was Hermann Nasse in 1836 who clearly defined that the sedimentation was correlated with the intensity of rouleaux formation. The subject was scarcely mentioned in the medical literature from 1850 until 1917 when Fahraeus noticed that oxalated blood removed from patients for chemical analysis and standing on the laboratory table waiting to be tested had settled in different proportions in the various bottles. He checked the patients from whom the blood had been drawn and found that most of them were post-partum women. In his enthusiasm, he thought that he had discovered a new test for pregnancy. As a scientist, he did not let his discovery rest and he soon found that there were other causes for the phenomenon. This led Westergren to devise the first commonly used modern technique for measuring the sedimentation rate.

Shortly after the introduction of the test into modern clinical medicine in Sweden, there was a surge of interest in its use. Hundreds of reports

of the change in the rate in various diseases were published and an untold number of variations of the test were introduced. It is Fahraeus, however, who deserves the credit for providing us with this simple but valuable means for following the course of illness.

We can safely say that like the clinical thermometer and the leukocyte count it has found its place as a guide to the physician in following the course of most organic disease. Certainly a rapid sedimentation rate is a sign that all is not well with the patient.

Erythrocyte sedimentation follows Stokes' well known physical law which governs the rate of fall of particles in a liquid medium. The rate of fall depends upon temperature, the size and specific gravity of the particles and the viscosity of the liquid. Since the viscosity of the plasma and the specific gravity of the erythrocytes are quite constant at all times, the only significant variable in the system is the size of the particle. Individual erythrocytes settle extremely slowly; however, the more they become aggregated into rouleaux, the more rapidly they fall because of the greater diameter of the aggregate particle.

The complex explanation of rouleaux formation is not known. Hematologists from John Hunter on have been curious about it. Why do erythrocytes align themselves into an orderly arrangement like stacks of coins? The answer is perhaps in the realm of physical chemistry. It is generally believed that the surface of the erythrocyte is water repellant in plasma and that the globulins, especially fibrinogen, augment this property. It can be shown simply that the sedimentation rate is almost zero in defibrinated blood or when erythrocytes are suspended in their normal concentration in serum albumin. Many hydrophilic colloids such as gelatin, agar, gum arabic and gum acacia greatly intensify rouleaux formation. What we know of the surface physics and chemistry related to this problem is beyond the scope of this paper. We will not have a complete answer, however, until we learn more about the erythrocyte surface.

The physiological significance of intravascular rouleaux formation is a subject of great interest. Shortly after Fahraeus described his discovery, Ploman showed that rouleaux would form in the retinal vessels if the circulation was slowed by putting pressure on the eye. Rouleaux or blood "sludge" as it is called by Knisely, can be observed in the capillaries of the conjunctiva of the eye when the sedimentation rate is increased. Such an obvious phenomenon in the capillary circulation would seem to have a meaning in pathology or physiology, but this does not seem to be the case. The problem is quite paradoxical when we realize that the healthy horse normally has a very rapid sedimentation rate.

I hope that with these remarks you will appreciate how a laboratory test became the foundation for medical theory and practice for over two thousand years and that the observations made today by medical technologists may in like manner open channels of thought for medical scientists which will bring them ever closer to a better understanding of physiology and pathology.

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THE DETERMINATION OF SERUM GLOBULIN BY THE BIURET METHOD*

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Introduction

In most clinical laboratories it is the practice to perform only the total serum protein and the serum albumin and to estimate the serum globulin value by subtraction. In the Biuret method of Ferro and Ham[1] the procedure for the total protein and the serum albumin is described, but again the globulin value is obtained by subtraction.

This paper describes a method for the actual determination of the globulin by the Biuret method of Ferro and Ham[1] by utilizing the globulin disc remaining after the albumin has been removed.

The advantage of performing the globulin test also, lies in the ability to check final results by the addition of the albumin and globulin values to equal the total protein value within an allowable error. A further advantage exists in being able to perform only the globulin test if that alone is requested and thereby saving time in doing extra unnecessary tests.

Procedure

Total Protein and Albumin

In the method described by Ferro and Ham[1] 0.5 ml. of serum is added to 9.5 ml. of 22.6% sodium sulfate solution, and after mixing 2 ml. is removed for the total protein and saved. To the remainder of the sodium sulfate-serum mixture two drops of aerosol and 2-3 ml. ether are added, the tube shaken well and centrifuged for five minutes. After centrifugation 2 ml. of the albumin layer is removed and saved.

Procedure for Serum Globulin

The remainder of the albumin layer and ether is discarded by decanting into another tube. The globulin disc is not discarded. The tube is then drained leaving the globulin disc undisturbed. The globulin disc is then dissolved by the addition of 7.5 ml. of 0.85% sodium chloride solution. Mix well. The sodium chloride solution not only serves to dissolve the globulin but is also used to reconstitute the globulin to its proper dilution. If 2 ml. of the sodium sulfate-serum mixture were not removed for a total protein determination, then the globulin disc must be reconstituted to volume by the addition of 9.5 ml. of 0.85% sodium chloride solution. Two ml. of the dissolved globulin is then used for the globulin test.

Procedure for Total Protein and A/G Ratio

1. Two ml. of 0.85% sodium chloride solution is used as a blank for all three determinations: the total protein, albumin and globulin.

2. Two ml. of solution now remains in the total protein tube and the albumin and globulin tubes.

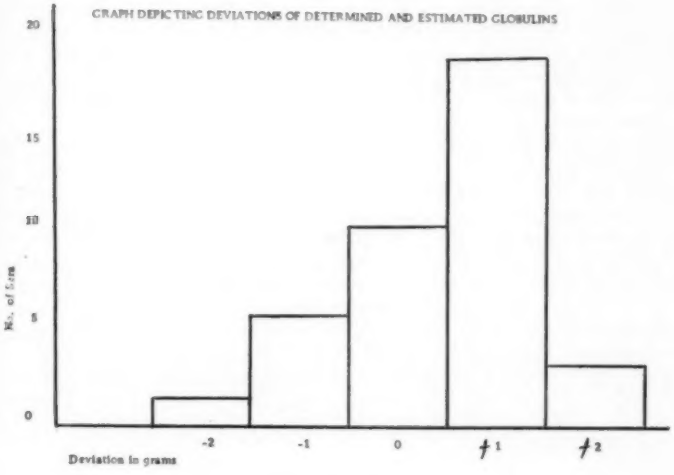
* Read before the 26th Annual Convention of ASMT, Milwaukee, Wisconsin, June, 1958.

3. To all tubes, the total protein, serum albumin, serum globulin and blank add 8 ml. of Biuret Reagent (3.0 gms. copper sulfate, 12.0 gms. sodium-potassium tartrate, and 2 gms. potassium iodide dissolved in 1000 ml. distilled water. Add 600 ml. of 10% sodium hydroxide and dilute to volume in a 2-liter volumetric flask). Mix by inversion.

4. Let stand 30 minutes at room temperature or 10 minutes at 30° C.

5. Read in percent transmission or optical density at a wave length of 540 mu. against the saline blank.

By this procedure the serum albumin plus the serum globulin should equal the total protein within approximately 0.1 gm. I have determined 37 sera for protein fractions by this method and have also determined the standard deviation for each fraction with excellent results. These tests were read in both the Beckman Spectrophotometer and the Leitz Colorimeter, although any good instrument should suffice.



Analysis of the Data

Out of 37 sera analyzed, 10 determined and estimated globulins were exactly the same. Eighteen determined globulins were 0.1 gm. higher than the estimated globulins and 5 determined globulins were 0.1 gm. lower than the estimated globulins, whereas only 4 determined globulins were 0.2 gm. higher or lower than the estimated globulins.

The next step was to determine the standard deviations by Dr. Bradley Copeland's formula, for the total protein, albumin, determined globulin and the estimated globulins. Therefore the last 10 sera were determined in duplicate and their standard deviations calculated.

TEST	STANDARD DEVIATION/100 ml. SERUM
Total Protein	±0.124
Albumin	±0.092
Determined Globulin	±0.05
Estimated Globulin	±0.071

The results obtained indicate that the standard deviation for the determined globulin is slightly smaller than the estimated globulin by 0.021 gms.

Summary

A method has been outlined utilizing the Biuret test in performing the actual determination of serum globulin instead of subtracting the albumin from the total protein and obtaining an estimated value for the globulin. The results indicate that the method is accurate and that the standard deviation of the determined globulin is slightly lower than the standard deviation of the estimated globulin. The difference between these two standard deviation values is insignificant.

By performing the determined globulin the accuracy of the total protein and albumin can be checked, if the albumin and determined globulin equal the total protein within the allowable error of 0.2 gm. The method is simple and can be performed along with the total protein and albumin, or independently if only the globulin is requested, by simply utilizing the globulin disc remaining after the albumin has been removed. If an error were made in either the total protein or albumin test then an incorrect estimated globulin value would be reported since the estimated globulin value can only be obtained by subtracting the albumin value from the total protein value. However by the use of the determined globulin test an error in either the total protein, albumin or even the globulin test would show up immediately, since the albumin and determined globulin values must equal the total protein value within an error of 0.2 gm.

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A RESUMÉ ON TRANSAMINASE*

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Enzyme chemistry, which has been for many years an important tool in the hands of the chemists in food, pharmaceutical, and industrial chemistry, gains more and more importance for the clinical laboratory. One of the newer enzymatic tests, which can serve as an important factor in the physician's diagnosis and prognosis is the determination of the serum transaminase

The transaminases are, as the name indicates, those of the many enzymes in animal tissue that transfer an amino group from one of several amino acids to its keto acid counterpart. They were first observed by Braunstein and Kritzman in 1938 in the breast muscle of the pigeon. Further work indicated that there are a great number of known amino-acids which undergo transamination and each one probably has a specific enzyme.

At the present time the clinical chemist is primarily concerned with two of these enzymes. They are the Serum Glutamic Oxalacetic Transaminase (SGO-T) and the Serum Glutamic Pyruvic Transaminase (SGP-T). These two enzymes show the following average distribution in the normal human tissue in units per gram wet tissue: (Dr. Wroblewski personal communication)

	SGO-T	SGP-T
Heart	155,000	7,130
Liver	142,400	43,800
Skeleton Muscle	99,300	4,750
Kidney	90,900	19,300
Pancreas	28,300	1,950
Spleen	13,600	1,210
Lung	10,000	668
Serum	20	15

The test could only be performed in research institutions or larger hospitals when it was first discovered in 1938. The work of Dr. Wroblewski and his co-workers of the Sloan-Kettering Institute, New York, improved the test so that it may be used in any laboratory at the present time. They found an elevation of the SGO-T in the serum of patients with a recent myocardial infarct. The degree varied with the extent of the infarct. (Fig. 1) These findings were confirmed by others.[1,2,3,5,6] It was observed that infarcts of less than one gram will cause a rise of the enzyme in the serum. Since the enzyme concentration of the infarcted muscle is substantially less than in the normal muscle tissue, it is assumed that the enzyme enters the blood stream through the damaged tissue.[5]

Ninety-five per cent of these cases with an acute myocardial infarction will show an elevated SGO-T. This increase may be 2-20 times above the normal depending upon the extent of the damage. The activity of the enzyme in the serum increases 4-6 hours after the attack, it reaches its peak in 18-36 hours and returns to normal in 3-6 days.[1,2]

The normal values are 8-40 units of SGO-T and 15-35 units of SGP-T.

*Received for publication April, 1958.

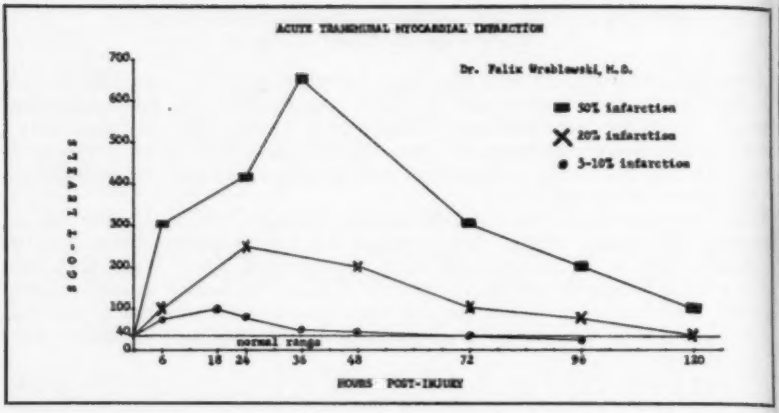


Fig. 1

Hemolyzed blood cannot be used since red cells contain larger quantities of transaminase than serum.

The test is more specific at times than the leukocyte count, the sedimentation rate, and the electrocardiogram in following a recent infarction. The transaminase level is normal in cases of arteriosclerosis, hypertensive cardiovascular disease, angina pectoris, active fibrillation, bundle branch block, old myocardial infarction, uremia, most cases of infarction of the lung, neoplastic conditions if there is no metastasis to the liver, and in many other conditions.[2,7]

An insignificant rise in the enzyme activity occurs after abdominal surgery. This is secondary to trauma to the striated muscles.

There is a very significant elevation in liver diseases. In hepatitis there is a marked increase and the elevation is usually higher in homologous serum jaundice. The level stays elevated 30-60 days before returning to normal value. An elevation occurs to a lesser degree in cases of obstructive jaundice. The activity returns to normal shortly after the obstruction has been corrected. The alkaline phosphatase and bilirubin do not return to normal as quickly as the transaminase.[4,7] (Fig. 2,3,4) The enzyme is probably excreted through the bile and any obstruction of the biliary tract will result in an elevation. These features will help to differentiate an obstructive jaundice from a hepatitis patient.

A simultaneous determination of SGO-T and SGP-T may help to differentiate between various disease states of the liver. In chronic disease states the SGP-T is less than SGO-T. In acute liver diseases the SGP-T is greater than SGO-T. In acute extra hepatic obstruction the SGP-T is greater than SGO-T.

It was found that the enzyme activity increases after death and that the increase is in a proportion to the length of time after death has occurred.[2]

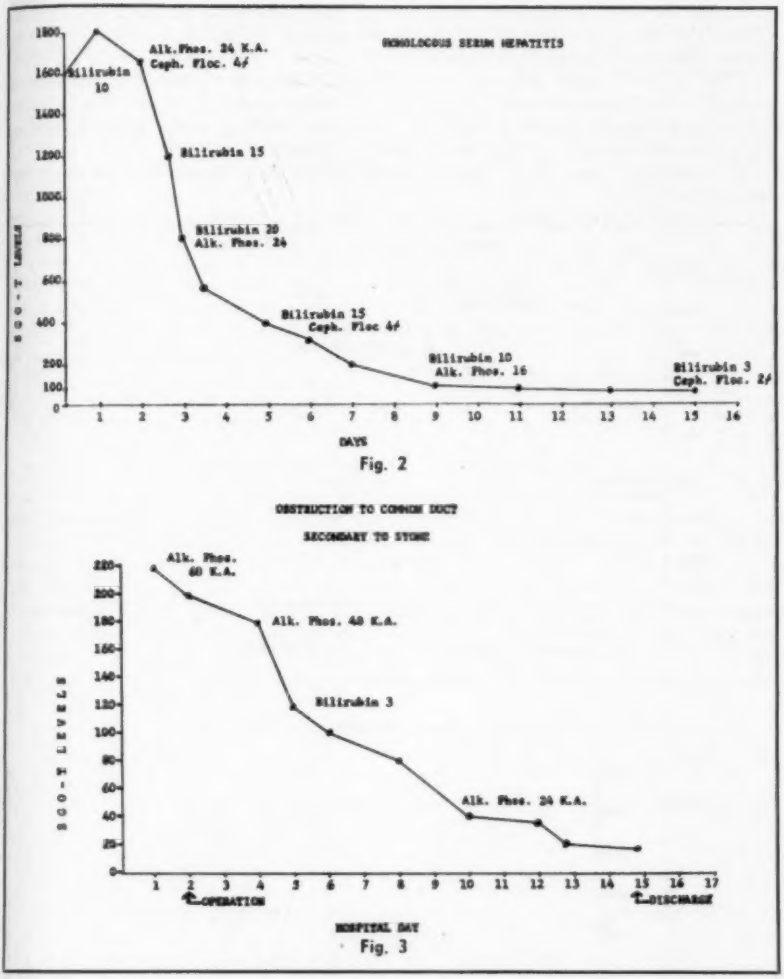


Fig. 2

Fig. 3

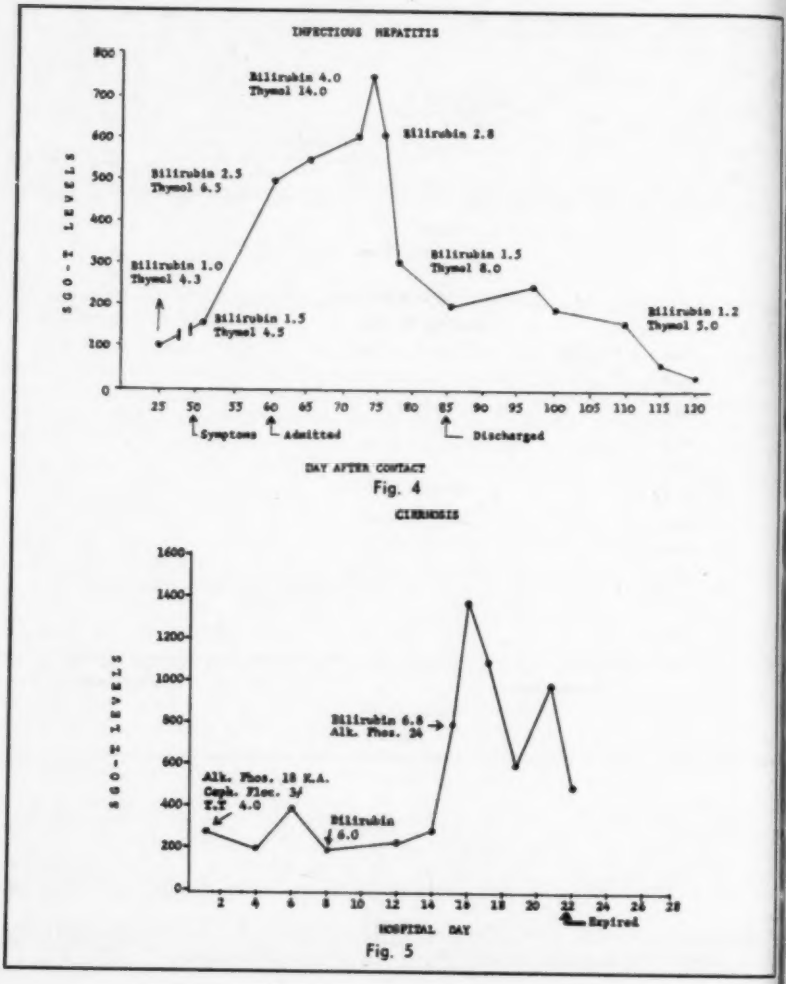
The transaminase level is increased when there is metastasis to the liver. This has not been found in lymphoma or leukemia. In all cases of liver metastasis the transaminase determination is a more sensitive indicator than the alkaline phosphatase and the Bromsulphothalein tests.

Bone tumors may be associated with an elevated alkaline phosphatase. The SGO-T is normal unless there are liver metastases.

There is also a marked increase of transaminase activity in carbon tetrachloride poisoning as might be expected. It is also increased in the active phase of cirrhosis. (Fig. 5)

The SGO-T may become elevated in 8-14 days after the patient is exposed to contaminated serum or the virus associated with hepatitis. Increased transaminase levels are observed before other liver function tests become positive. (Fig. 4—Case of a Blood Donor)

We employ this test in our blood bank when a donor has an elevated thymol turbidity.



Tuberculosis patients are treated with pyrazinamide. This drug may cause a toxic hepatitis. Serial determinations of transaminase and other liver functional tests are done in these cases. The SGO-T becomes elevated 3 weeks before any other test becomes positive in most cases.

The human brain tissue contains large amounts of transaminase. Lesions of the central nervous system do not show an elevation of this enzyme. It is believed that some blood barrier to the enzyme exists.[12] At the present time, research is going on to establish normal values for spinal fluid, and also the level in various central-nervous-system lesions as determined in spinal fluid.[13]

The enzyme activity in healthy persons is unchanged from day to day. The serum transaminase level remains stable at least 4 days if the serum is kept refrigerated between 0 and 5° C. One transaminase unit is defined as a decrease in optical density of 0.001 per minute per milliliter of serum under standardized conditions.

The activity of SGO-T and of the SGP-T can be determined by:

1. Paper Chromatography
2. Spectrophotometry
3. Colorimetry

Since in most clinical laboratories no instrument for paper chromatography is available, nor spectrophotometers with a wavelength of 340 mu., the colorimetric method is the procedure of choice. The first new procedure, which also has the advantage that it does not require unstable enzymes as reagents, was developed by Dr. Cabaud, Dr. Leeper, and Dr. Wroblewski,[8,9] based on an earlier method of Tonhazy, White, and Umbreit.[10] There are other practical colorimetric methods as the methods by Sigma-Frankel and by Dade which are used in many laboratories and they compare well with the original spectrophotometric method. The colorimetric procedures allow a determination at a wavelength between 490-510 mu.

SGO-Transaminase Assay Methods

Chromatographic

$$\text{Asparate} + \text{alpha-keto glutarate} \overset{\text{SGO-T}}{\rightleftarrows} \text{glutamate} + \text{oxalacetate}$$

formation of glutamate after 3 hours

Spectrophotometric

$$\text{Asparate} + \text{alpha-keto glutarate} \overset{\text{SGO-T}}{\rightleftarrows} \text{glutamate} + \text{oxalacetate}$$

$$\text{Oxalacetic} + \text{DPNH} + H^{+} \overset{\text{malic dehyrrogenase}}{\rightleftarrows} \text{malate} + DPN^{+}$$

rate of disappearance of $DPNH_2$

Colorimetric

$$\text{Asparate} + \text{alpha-keto glutarate} \overset{\text{SGO-T}}{\rightleftarrows} \text{glutamate} + \text{oxalacetate}$$

(Oxalacetate→ pyruvate→)

formation of pyruvate after 20 minutes

The main difference in the spectrophotometric and the colorimetric methods is the following: In the colorimetric method the micrograms of the formed pyruvate is the unit measurement for the SGO-T and the

SGP-T activity.

In the spectrophotometric method the rate of the transamination reaction is determined by the rate of the disappearance of $DPNH_2$ a result of the enzymatic action. The colorimetric method measures the end-product of the transamination and the spectrophotometric the rate of transamination. Both methods are therefore not directly comparable, but the activity values obtained in the colorimetric method roughly approximate those of the spectrophotometric method. The colorimetric method is simpler and is used by most everyone for routine purposes.

It is sufficient, for most clinical purposes, to determine only the SGO-T activity. The SGP-T is elevated in myocardinal infarction, only if there is a substantial necrosis of the tissue.

Summary

The activity of SGO-T and SGP-T is important in diagnosing myocardinal infarction and estimating the extent of the damage. It is also useful in diagnosing liver diseases. It will help to differentiate a hepatic from an obstructive jaundice. It may indicate the presence of metastatic lesions in the liver when all other tests are normal.

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PREPARING EGG BASE MEDIA FOR TUBERCLE BACILLI IN THE AUTOCLAVE*

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Introduction:

The routine method of preparing egg base media for tubercle bacilli, used in most laboratories, is slow and time consuming. The process is accomplished only after about 50 minutes to 90 minutes inspissation and sterilizing time. In addition, a period of 2 hours to 3 hours is necessary to heat the inspissator to the desired temperature. In a laboratory doing a large volume of work it is desirable to have a rapid method in order to produce enough media. This method must not only be rapid but must also be very reliable. The purpose of this paper is to describe a rapid method of preparing egg base media for tubercle bacilli. The method utilizes the conventional autoclave with pressure regulator.

Method:

1. Adjust the pressure to 9 pounds in autoclave with adjustable pressure. This is equivalent to 114.3° C.
2. Place pans of media in autoclave with the desired slant.
3. Close all valves. Make sure that all cold water from condensed steam is out of the steam line.
4. Open steam valve and allow pressure to rise slowly to the designated 9 pounds.
5. Time media for 10 minutes, which is the coagulation time.
6. Slightly open the air escape valve, which is usually at the top and allow the air in the chamber to escape. Do not allow the pressure to fall more than 1 pound during this procedure. This operation will take about 5 minutes.
7. Close air escape valve and time media for 15 minutes. This is the sterilization time.
8. Close steam valve and let pressure fall slowly. If the pressure has not fallen to zero in ½ hour, open the water outlet valve at the bottom of autoclave slightly. When pressure has fallen to zero open the water outlet valve slowly, then all other valves.

Discussion:

The modern autoclave is provided with a pressure regulator, which makes it possible to regulate the pressure of the autoclave. By regulating the pressure the heat is also regulated. Some media contain components which would be rapidly destroyed at 121° C. but can withstand somewhat lower temperatures. These media are sterilized by exposure to free-flowing live steam at atmospheric pressure on three successive days in an Arnold Sterilizer. This fractional sterilization or Tyndallization destroys the vegetative cells and the spores germinate during the storage

* Received for publication November, 1958.

interval. This procedure is only applicable to nutrient media that will promote germination. The inspissation method at 85° C. of egg media for tubercle bacilli is a modification of fractional sterilization. Boiling water and free-flowing steam never reach a temperature above 100° C. Boiling takes place at the temperature at which the pressure of saturated vapor is equal to the pressure on the surface of the liquid. The pressure on the surface of the liquid depends on the height. In mountainous regions water boils at a lower temperature because the higher you go the less atmosphere you have above you and the less surface pressure it takes to equalize the atmospheric pressure. By contrast an autoclave contains steam under pressure. The higher the pressure the higher the temperature. Steam under pressure is therefore hotter than boiling water or free-flowing steam such as is used in an electric inspissator, etc. As a result of being hotter the operation can be accomplished faster in the autoclave if the heat is controlled.

Steam hydrates and thus promotes coagulation and it also produces hydrolysis at autoclave temperature. Air cannot do this. Therefore, in autoclaving, the dry air must be excluded before the steam pressure is allowed to rise, preparatory to sterilizing. Air mixed with steam will lower the temperature and not afford a temperature sufficient for sterilizing. In the method described the steam and air are allowed to mix for the 10 minutes of coagulation time, which is the initial procedure. This will maintain a sufficient temperature for coagulation. However, for the 15 minutes of sterilization time, all the air has to be excluded in order to attain the necessary temperature for sterilization.

The following points are the important precautions that must be taken:

1. Make sure all valves are closed at the beginning or the media will blow or puff up.

2. During the "bleeding period" or period for the exclusion of air from the chamber, the pressure should not fall more than 1 pound. If the pressure falls more than 1 pound the media will blow or puff up. On the other hand, if all the air is not excluded from the chamber, the media will not be sterilized. This valve should be opened just wide enough to allow the pressure to fall 1 pound in 5 minutes.

3. The pressure should not be allowed to go over 10 pounds or 115.6° C. Temperatures above 10 pounds will cause the malachite green to break down and it will lose its color. Consequently the media will lose its selectivity.

4. Do not open the autoclave door before opening the water drain valve, as the water will run out on the floor that has accumulated in the bottom of the autoclave. This water accumulates from condensed steam. The water drain valve is usually at the bottom of the autoclave.

5. Finally the media should not remain in the autoclave longer than thirty minutes after having been sterilized in order not to reduce its selectivity.

Conclusion:

The average inspissator holds two pans of about the size, 13¼ x 11½ x 4½ inches. Each pan will hold about 150-160 tubes of media. The average

autoclave will hold four of these same sized pans, which means that twice as much media can be tubed at the same time. The time required with the average inspissator is 50-90 minutes, excluding the time that it takes to reach the desired temperature. Add this time and the process will take at least 2-3 hours. The inspissator is allowed to heat up first and then the full pans are placed in it. This lowers the temperature and this extra time is required to bring the whole system to the proper temperature. The operator cannot begin to time the media until the required temperature is reached. This is eliminated in the autoclave because the system is rapidly heated to the desired temperature. The whole process takes about 1 hour, including the time for letting the pressure go down to zero. The method described is therefore faster, more economical, and especially geared to "a large volume of work situation." Over a period of 18 months in our laboratory egg base media for tubercle bacilli, made in the autoclave, has compared favorably with the inspissator made media in all properties. By using this method an employee has been released to do other things in the laboratory.

Summary:

A rapid method for coagulating and sterilizing egg base media for tubercle bacilli in an autoclave equipped with a pressure regulator, has been described. The method described permits twice as much media of comparable quality to be made in about ⅓ or less the time that is necessary with an ordinary inspissator.

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ABSTRACTS

OX CELL HEMOLYSIN TEST AS A DIAGNOSTIC PROCEDURE IN INFECTIOUS MONONUCLEOSIS

William Mikkelsen, C. J. Tupper, and Jean Murray. (University of Michigan, Health Servies and Hospital, Ann Arbor, Mich.) Jour. Lab. Clin. Med. 52, 648-652 (1958).

The results of two hundred and eight serum specimens from 183 patients with presumptive diagnosis of infectious mononucleosis is presented in which the heterophil antibody test is compared with the ox cell hemolysin test. In the heterophil antibody test the serum was routinely adsorbed with guinea pig kidney antigen as described by Davidsohn. In the ox cell hemolysin test beef cells have been found to behave in a fashion identical to the less readily available ox red blood cells in the hemolysis procedures.

The authors consider a heterophil agglutination titre of 1:56 as negative, 1:112 to 1:224 as suspicious and 1:448 or greater as positive. For comparative purposes an ox cell hemolysin titre of 1:48 or less is considered as negative, 1:96 to 1:384 as suspicious and 1:796 or greater as positive. Results of the two procedures have been carefully evaluated in the 72 patients with definite infectious mononucleosis and 92 patients without infectious mononucleosis. In the group with definite infectious mononucleosis the over-all incidence of positive results with ox cell hemolysin was greater by 75 per cent as compared to the 52 per cent positive results in the heterophil agglutination test. The ox cell hemolysin test was more frequently positive during the first and second weeks of illness than the heterophil test.

Technically, the ox cell hemolysin titre test has been found to be simpler, faster, and less expensive to perform than the heterophil agglutination test. It is suggested that the greatest diagnostic assistance will be obtained by performing both tests rather than either procedure alone.

AN IMPROVED METHOD FOR ISOLATING VIABLE HUMAN LEUKOCYTES FROM PERIPHERAL BLOOD

Joseph H. Lapin, Andrew Horonick, and Robert H. Lapin (Institute of Geriatrics, Div. of the Home and Hospital of the Daughters of Jacob, Bronx, N. Y.) Blood, 13, 1001-1005 (1958).

The method described is an effort to separate leukocytes and prepare a suspension in which the ratio of erythrocytes to leukocytes is considerably less than that obtained with previously described technics. It is necessary to siliconize all glassware and use Arquad treated needles. Two 10 ml. samples of blood are prepared, one test tube contains heparin and the second test tube contains heparin and 6% Dextran in saline. The plasma from tube one is saved in order to resuspend the sedimented cell-button from the second tube. The Dextran treated blood is allowed to stand at 37° C. for 60 minutes and the plasma transferred and centrifuged and resuspended in isotonic saline and stands another 60 minutes. This is repeated and then the cell-button is resuspended in the plasma from tube one.

Leukocyte and erythrocyte counts were performed after each step. The sedimentation of the erythrocytes with Dextran results in a determination of RBC:WBC ratio from the 1000:1 of peripherial blood, to 2.34:1. After sedimentation in isotonic in isotonic saline, the RBC:WBC ratio is decreased to 0.16:1. Due to the decrease of erythrocytes by this method, it should prove valuable in preparin leukocytes for immunologic studies.

WHOLE BLOOD TRANSAMINASE LEVELS IN ANEMIA

Martin Sacs, Paul W. Spear. (VA Hospital, Brooklyn, N. Y.) Jour. Lab. Clin. Med. 51. 926-933 (1958).

This report is concerned with the presentation of data on the glutamic oxalacetic transaminase (GOT) variations during periods of increased marrow activity associated with red cell proliferation. Twenty-two patients with severe chronic anemia, and 19 patients with a variety of hemolytic anemias were studied and the GOT activity of their blood was compared to a group of normal patients. The results indicate that the mean transaminase activity for the group with hemolytic anemias is approximately 2 to 2½ times greater than the control group. An attempt was made to correlate the production of reticulocytes with the GOT activity in the hemolytic anemias.

Normal GOT levels in two cases of melagoblastic anemia increased markedly after B_{12} therapy and were maintained at this level despite the return of the reticulocyte count and hemoglobin to normal. The authors suggested that measurements of whole blood GOT activity may be useful as an indicator of bone marrow activity.

AN IMPROVED METHOD FOR THE CHEMICAL DETERMINATION OF URINARY CATECHOL AMINES

Roy B. Johnson, Jr. (Div. of Laboratories, Scripps Clinic and Research Foundation) Jour. Lab. Clin. Med. 51, 956-963 (1958).

The value of the urinary catechol amine determination as an aid in the diagnosis of pheochromocytoma is becoming increasingly apparent. Several chemical methods have been described in which the catechol amines are adsorbed on alumina or alumuim hydroxide by either the column or batch method.

The alumina column method described in this report is a modification of those of Lund, von Euler et. al., and Goldenberg. Two important advantages of this modification are that the urine samples are prepared so that they flow rapidly through the alumina column and that a better means of discriminating the fluorescence of the catechol amines is provided.

The advantages of the proposed method are the incorporation of a rapid column isolation provided with a fluorometric analysis which permits increased reliability of the results. Addition of oxalic acid to the collection bottle solved the precipitate problem and the urine is filtered free of precipitate at an acid pH where the catechol amines are relatively stable.

The normal values by this method are substantially higher than reported by other investigators. This is probably due to the oxalic acid since it does protect epinephrine under oxidizing conditions. This modification reduces extraneous fluorescence and standardizes the procedure with an increase specificity in the fluorescence analysis obtained. The method has a reproducibility of approximately ±10 per cent in the normal range. Total catechol amines excreted as norepinephrine averages 169 ug per 24 hours ± S.D. 73 by this method.

CONGENITAL HEMOLYTIC ANEMIA WITH ABNORMAL PIGMENT METABOLISM AND RED CELL INCLUSION BODIES: A NEW CLINICAL SYNDROME

Robert D. Lange and Joseph H. Akeroyd. (Dept. of Hematology, Walter Reed Army Medical Center, Washington, D. C.) Blood: 13, 950-958 (1958).

A case report and special studies on a 14-year-old girl with congential hemolytic anemia is reported in which 14 per cent of her erythrocytes contained unusual inclusion bodies. The inclusion bodies are described as a light blue-gray, irregularly shaped bodies which vary in size up to 2 u as seen in Leishman-Giemsa stained blood films. A faint ring of tiny granules could be seen around the periphery of some of the inclusion bodies and the Prussian blue reaction accentuated this ring. On electron microscopy the inclusions appeared as relatively dense structures. They are partially soluble in methanol, insoluble in distilled water, Feulgen-negative, and were not destroyed by freezing.

The inclusion bodies do not resemble Pappenheimer bodies, they are not siderin, they resemble Heinz bodies, however; they are visible when stained with Romanowsky dyes, whereas Heinz bodies are visible only by use of vital dyes or dark field microscopy. It is believed that the inclusion bodies are probably caused by an inborn error in erythrocytic metabolism.

In addition, the child has been known to pass a dark brown or blackish colored urine since the age of 30 months and that this pigment probably belongs to the bilifuscin and mesobilifuscin group.

AEC REPORT DESCRIBES BLOOD VOLUME DETERMINATION BY RADIOACTIVITY

Techniques involved in the determination of blood volume and its fluctuations with radioactive isotopes are discussed in a report of recent research for the Atomic Energy Commission just released to the public through the Office of Technical Services, U. S. Department of Commerce. A study of the use of the Index of Cardiac Clearance for measuring cardiac output also is described.

Part 1 of the two-part report deals with the principle of blood determination with radioactive substances and the calculations involved. The Plastic Coil technique for radioactivity analysis is described, and the method is applied to blood volume determination with sodium chromate-tagged cells, RISA (R), and a combination of the two.

Among results of the radioactivity studies of blood volume fluctuations, it was shown that total volume may increase as much as 500 milliliters following ingestion of food and fluid, and from 400 to 500 milliliters with administration of Dextran. The studies also included vasodilation and vasoconstriction, removal of 25 percent of blood volume, and serial measurements of volume fluctuations. An analog computer was devised for automatic calculation of dilution.

Research described in the second part of the report concludes that the Index of Cardiac Clearance may well develop into a practical and simple method for evaluating cardiac functions and the effects of drugs on the normal and diseased myocardium. A conversion table enables calculation of approximate cardiac output value.

The report is AECU-3614 *Part 1—Blood Volume Determinations with Radioactive Isotopes and Observations on Blood Volume Fluctuations; Part 2—Index of Cardiac Clearance*, George Washington University for U. S. Atomic Energy Commission, Mar. 1958, 49 pages. It may be ordered from OTS, U. S. Department of Commerce, Washington 25, D. C., price $1.

Questions and Answers

ON BLOOD BANKING PROBLEMS

Edited by NED G. MAXWELL, M.D.

Milwaukee, Wisconsin

At the 1957 national meeting of the American Society of Medical Technologists, Drs. Grifitt's, Greenwalt and Stern were asked a series of current questions. A selected few of the questions and answers are here published from the AABB Bulletin, Feb., March, and May, 1958, under the name of the panelist.

Q. The statement has been made that blood for an infant should be cross-matched with its mother's serum. Does this hold in all cases?

A. This practice should be followed in all cases during the newborn period. This is because the infant does not have its own iso-agglutinins. The only antibodies detectable in the infant's serum during the newborn period are those which have been transmitted to it from its mother. For this reason, any unexpected isoagglutinins will usually be in much lower titer in the infant's serum than in the maternal serum, and this makes compatibility tests unreliable and, in most instances, useless unless the mother's serum is employed. When this recommended procedure is employed, it will of course always be necessary to match blood which is compatible in the ABO system with the serum of the mother. A few examples of the problems which arise will be helpful in illustrating the principles involved. If the infant is group A and its mother is group A, one should preferably use group A blood which must be compatible with any other isoagglutinins present in the mother's serum. If the mother is group AB, it is possible to crossmatch blood of the same group as the infant's with the mother's serum. If the mother is group O and the infant either A or B, only group O blood can be crossmatched. This is very important in hemolytic disease which is due to ABO incompatibility, for in these cases one must use group O blood and not blood of the same ABO group as that of the infant.

T. J. Greenwalt, M.D.
Milwaukee, Wisconsin

Q. Should the Coombs test be read with the naked eye, with the help of a hand lens, or microscopically?

A. The Coombs test can be performed in several different ways. In the slide technic, a 40 to 50 percent suspension of the well-washed cells is employed and the test performed either at room temperature or on a heated viewing box. The heavy suspension of red cells used in this technic precludes microscopic interpretation.

It is my impression that the tube test is more widely employed in this country. In this test a 2 to 5 percent suspension of the well-washed red cells is used, and it is the general rule to check all macroscopic readings including that of the Coombs test microscopically. The use of a suitable hand lens is perfectly acceptable in experienced hands. It is just as simple and perhaps better to place the tube which has been read negative macroscopically under the medium power (10X) objective of the microscope. The suspension of red cells can be readily visualized if a 10X ocular is used in combination with this objective (magnification x 100).

It is most important to emphasize that suitable negative and positive controls should be included with every serologic test performed in the laboratory. It is essential to know that the reagent which is being employed gives clear-cut reactions with known positive cells and does not give false positive reactions with known negative cells. One must be especially careful when doing indirect Coombs test with enzyme-treated cells.

T. J. Greenwalt, M.D.
Milwaukee, Wisconsin

Q. When anti-A and anti-B isoagglutinins in the serum of a person of group O have been completely neutralized by addition of group-specific (Witebsky) substance, sometimes agglutination is obtained when the "neutralized" serum is incubated with A or B red cells and subsequently an indirect antiglobulin (Coombs) test is done on the red cells. What is the interpretation of these findings?

A. This technic has been proposed by Grove-Rasmussen et al (Am. J. Clin. Path.: 23:828, 1953) as a method of performing the minor crossmatch whenever a universal donor is to be used. In this way, assurance is gained that isoagglutinins are not present in excessive

quantities or with dangerous qualitative properties ("immune" antibodies). The test is based on the fact that group-specific substance is not capable of neutralizing the "immune" anti-A and anti-B antibodies. The indirect antiglobulin technic is especially suitable for demonstrating agglutination by "immune" anti-A or anti-B, even when saline-suspended red cells are not agglutinated. In conclusion, the failure of group-specific substance to neutralize anti-A or anti-B, as shown by the anti-globulin test, should cause rejection of such blood for transfusion outside of its own ABO group.

Kurt Stern, M.D.
Chicago, Illinois

Q. Twice recently, in our hospital, we have had to use an O donor for a recipient of another type, one of group A, the other of group B. In both cases, the minor crossmatch showed no clumping. Why? Both patients and the donors were retyped several times in order to exclude any error.

A. The absence of clumping may well have been due to a very low titer of the anti-A or anti-B agglutinin in the donor's serum which on further aging of the serum in the pilot tube may have dropped below the level of detectability. I presume that in processing the donors "reverse" or confirmation grouping was carried out and that at that time the normally expected isoagglutinins were detected. In the case of the patient of group A, there was also the possibility that he may have belonged to subgroup A_2. A_3, etc., which in combination with the low and poorly preserved anti-A could have explained absence of agglutination.

Kurt Stern, M.D.
Chicago, Illinois

Q. Recently I tested an obstetrical patient who had suffered a severe hemorrhage. I was unable to determine her Rh; so naturally I gave her Rh negative blood. The next morning, with a fresh specimen and also with the specimen collected the night before she was a clear Rh positive. I would like to know why the test was not positive at first.

A. The technic used for testing the Rh factor is not clear from the information supplied. It simply states that the technologist was unable to determine the Rh; the following morning there was a clear Rh positive reaction. There are many reasons for errors in Rh typing; usually with good, strong, and specific serums the technics recommended by the manufacturer will give clearcut results. However, for example, if heat is required in the test and it is omitted, one might get a negative reaction. If one is doing a slide test with a specimen of blood which is quite anemic, weak agglutination or even no agglutination may result; whereas clearcut positive reactions may occur when adequate numbers of red cells are mixed with the serum on the slide where heat is provided. By the same token, a very rapidly performed tube test with serum which is not designed for that purpose may give negative reactions. Rh testing, like any other laboratory procedure can best be done when following the directions recommended for the use of diagnostic reagent. If these were clearly followed and a negative reaction was received with Rh positive blood, then one must look to the quality of the blood specimen or some other error in technic. The labeling or other causes which do not result from faults in technics should be checked also.

James J. Griffitts, M.D.
Miami, Florida

Q. Does the saline crossmatch used in conjunction with the Coombs crossmatch pick up any antibody Coombs alone does not?

A. If one will refer to the papers of Ellis, Stern, and others, it will be apparent that there are some antibodies which are picked up better in saline and more particularly in high protein media than by indirect Coombs tests. Occasionally the saline test may give a positive reaction when the Coombs test is negative. For example, the Le^a antibody may be eluted from the cells in the washing process and give negative reactions. The concept of a saline crossmatch followed by an indirect Coombs crossmatch as being the ultimate in safety is incorrect. I feel that the high protein crossmatch on slides and in tubes is capable of picking up many things that make it unnecessary to carry out the indirect Coombs test routinely, since the antibody will be quickly demonstrated before it is necessary to confirm by indirect Coombs test. However, the indirect Coombs test will occasionally be positive when these two methods are negative. I think the saline crossmatch should never be done alone nor do I feel that the saline plus the Coombs is as good as the high protein crossmatch plus Coombs.

James J. Griffitts, M.D.
Miami, Florida

Q. We desire to make or purchase an alarm for our blood bank refrigerator in which the criterion would be not the air temperature of the refrigerator but the temperature of liquids stored in bottles in the refrigerator. Would a thermocouple immersed in a bottle of water or electrolyte solution answer this purpose?

A. I have had no personal experience with the use of alarm systems which are controlled by the temperature of liquids stored in the refrigerator. This is because I firmly believe that controlling the temperature of a refrigerator in this manner is wrong. The reason for this is that setting up the mechanism in this manner creates a lag period which may amount to hours because the alarm system will not be set off until the temperature of the liquid in the bottle has reached the

critical value. By this time all the blood in the bottles in the refrigerator will have also reached this critical value. When the temperature of the circulating air is controlled by a well-placed bulb the alarm will be set off long before the temperature of the solutions has risen to the critical level.

I do not mean to imply that it is not wise to know the temperature inside containers stored in a refrigerator. This can be easily accomplished by keeping a stoppered bottle of water with a properly calibrated thermometer in the refrigerator at all times.

T. J. Greenwalt, M.D.
Milwaukee, Wisconsin

Q. In group O patients, when A and B isoagglutinins have been completely neutralized with Witebsky substance and subsequent Coombs test with A and B cells as antigens are positive, what is the interpretation?

A. When A and B isohemagglutinins have been completely neutralized with Witebsky substances and subsequent Coombs test with A_1 and B cells are positive, it usually means that the individual has immune forms of anti-A and anti-B antibody.

James J. Griffitts, M.D.
Miami, Florida

Q. In our blood bank we have 3-6 cases per week of hemolytic disease of the newborn due to ABO incompatibility. Some cases are tentative and some obvious. Our findings show maternal titers in saline of 1:400 and up, and incompatible agglutinins as frequently demonstrated in the infants' sera by indirect Coombs and/or albumin tube test. I would like an opinion as to whether in such cases it is worthwhile to examine for possible antibodies outside the ABO system.

A. In the diagnosis of ABO hemolytic disease of the newborn, the prenatal or postpartum findings in the maternal serum are, as a rule, of little help. The most consistent finding is the presence of hemolytic antibody (anti-A or anti-B, respectively) of considerable activity. However, the presence of this hemolysin by no means prognosticates or indicates the presence of actual clinical disease in the infant. On the other hand, its absence immediately postpartum is sometimes observed with the hemolytic antibody first appearing two to three weeks after the delivery.

The most reliable laboratory finding at the time of birth is the presence of the incompatible agglutinin in the serum of the infant. In our experience, both indirect antiglobulin technics and the use of papain-treated red cells should be employed, since in some instances one, and in other instances the other technic is more sensitive.* However, in evaluating the presence of this incompatible agglutinin, one must keep in mind that it may be associated with subclinical disease or even be present without any disease of the newborn. In our own series such was the case in approximately ten percent of infants born in heterospecific pregnancies. Hence, the actual decision for treatment must be based on the further clinical course and laboratory findings, particularly the rise in serum bilirubin and the time of appearance and degree of clinical jaundice.

In any event, it is mandatory to exclude the presence of any other form of isosensitization in the mother. A strong positive Coombs test on the infant should, in my opinion, make one suspicious of presence of isosensitization other than to the A or B factor. One also must take into consideration the possibility, though of rare occurrence, of double isosensitization, i.e. to the A or B and another antigen.

In the event that a replacement transfusion is required, the safest procedure is provided by using for the major crossmatch (preferably indirect anti-globulin technic) the serum of the mother with the red cells of the prospective donor for the replacement transfusion. In this way one will be able to detect any incompatibility outside of the ABO system. Additional methods of excluding the presence of isosensitization to antigens other than A or B consist of: 1) screening the maternal serum with panel cells of known antigenic composition; and 2) absorption tests of the maternal serum with: a) the red cells of her husband, and b) with red cells from another donor of the same ABO group as the husband and differing from the husband in antigens such as Kell, Duffy and Kidd.

Kurt Stern, M.D.
Chicago

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* Stern, K. Davidsohn, I. and Buznitsky, A. "Neonatal Serologic Diagnosis of Hemolytic Disease in the Newborn Caused by ABO Incompatibility." J. Lab. and Clin. Med. 50:550, 1957.

BREATH ANALYSIS METHOD TESTS FOR CARBON MONOXIDE POISONING*

Two reports of Air Force research into the problem of carbon monoxide poisoning, one describing a rapid method for testing for CO by breath analysis and the other discussing inaccuracies in the technique of extrapolating presumed *in vivo* blood carbon monoxide levels, have just been released through the Office of Technical Services, U. S. Department of Commerce. Three other USAF reports of medical and human engineering research also are available. The volumes are:

An Estimation of Exposure to Carbon Monoxide by Breath Analysis. P. E. Sturrock and G. Kitzes, Wright Air Development Center, U. S. Air Force. Mar. 1958. 14 pages. (Order PB 131828 from OTS, U. S. Department of Commerce, Washington 25, D. C., 50 cents.) A semiquantitative screening method for determination of carbon monoxide poisoning in human subjects is presented. The method is based upon measurement of the carbon monoxide concentration of the breath with the National Bureau of Standards colorimetric carbon monoxide-indicating gel, a substance used for air analysis. The results of a "smoker-nonsmoker" survey of 54 subjects are included to show the significance of the method in measuring incipient carbon monoxide-exposure levels. The method is said to be accurate to two percent blood saturation. It is rapid, easy to use, and supplements the use of blood samples.

Postmortem Carbon Monoxide Analysis: Significance of Tissue Blood Content. A. Tamas and J. McElroy, Wright Air Development Center, U. S. Air Force. Nov. 1957, 19 pages. (Order PB 131725 from OTS, U. S. Department of Commerce, Washington 25, D. C., 50 cents.) Proper interpretation of the results of a postmortem tissue analysis for carbon monoxide is essential to investigations of major aircraft accidents. The pitfalls and shortcomings of the present technique of extrapolating presumed *in vivo* blood carbon monoxide levels from data obtained by rat experimentation are described. Data are presented which indicate the necessity for relating carbon monoxide tissue analyses to the tissue blood content.

The Effects of Stress on Uropepsin Excretion. R. H. Bonner, Wright Air Development Center, U. S. Air Force. Dec. 1957. 11 pages. (Order PB 131708 from OTS, U. S. Department of Commerce, Washington 25, D. C., 50 cents.) A preliminary investigation of uropepsin changes in simulated flight stress is described. Twenty-three subjects were tested under conditions of prolonged positive G, crew confinement, exposure to high temperature-high altitude, and visual and auditory deprivation. Uropepsin changes are reported and an effort is made to interpret and evaluate them. Modifications of the assay technique are also discussed

A Miniature, Direct-Plotting Pulse-Frequency Nomogram. K. M. Chapman, Wright Air Development Center, U. S. Air Force. Nov. 1957. 9 pages. (Order PB 131672 from OTS, U. S. Department of Commerce, Washington 25, D. C., 50 cents.) A device is described for conveniently and inexpensively plotting event rates from time-based data records,

* From the UNITED STATES DEPARTMENT OF COMMERCE, Office of Technical Services, September, 1958.
USCOMM-DC-54071

such as heart rate from electrocardiograms, and discharge frequency from volleys of nerve impulses. Principles of design, construction of a prototype, and photographic duplication of copies in quantity are discussed. The use of the nomogram for plotting nerve impulse frequencies is illustrated.

A Study of Muscle Forces and Fatigue. P. A. Hunsicker, University of Michigan for Wright Air Development Center, U. S. Air Force. Dec. 1957. 54 pages. (Order PB 131722 from OTS, U. S. Department of Commerce, Washington 25, D. C., $1.50.) The purpose of this research was to determine the strength which an individual can exert with his arms and wrists while seated in a simulated pilot seat. The results, which are important in the design and placement of hand levers, are presented in percentile tables and graphs. Several recommendations are offered.

MEDICAL TECHNOLOGISTS WANTED

Medical Technologist—Positions available in 300 bed medical center, Northern New England College community in year-round resort area. Starting salary open. 40 hour week, no night call. Liberal fringe benefits. For further information write: Director of Laboratories, Mary Hitchcock Memorial Hospital, Hanover, New Hampshire.

Clinical Laboratory Technologist, ASCP or eligible. Liberal vacation, sick leave, no call. Salary depends upon qualifications. 400 bed hospital. Contact Personnel Office, Iowa Methodist Hospital, Des Moines, Iowa.

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Male Frogs for Pregnancy Tests: One dozen $3.50, or $3.00 per dozen for 2 dozen or more. Mealworms (food), thousand $3.35. Postage extra. Frog Bulletin and Scientific Supply Catalog on request. Quivira Specialties Co., 4204 W. 21st St., Topeka 32, Kansas.

Wanted: Two (2) female medical technologists, ASCP certified or eligible—200 bed general hospital; excellent salary and personnel policies; paid one month's vacation, holidays and sick leave; modern laboratory, air-conditioned. Apply Mr. W. A. Towle, Jr., Administrator, Bristol Hospital, Bristol, Connecticut.

Medical Technologist, MT (ASCP). Science Degree Exp. Rotating service. General, 250 bed, non-denominational hospital. No night call. 40 hr. wk. Vacation—4 wks. 20 min. to N. Y. C. Apply Asst. Lab. Dir., St. Barnabas Medical Center, 685 High St., Newark 2, N. J.

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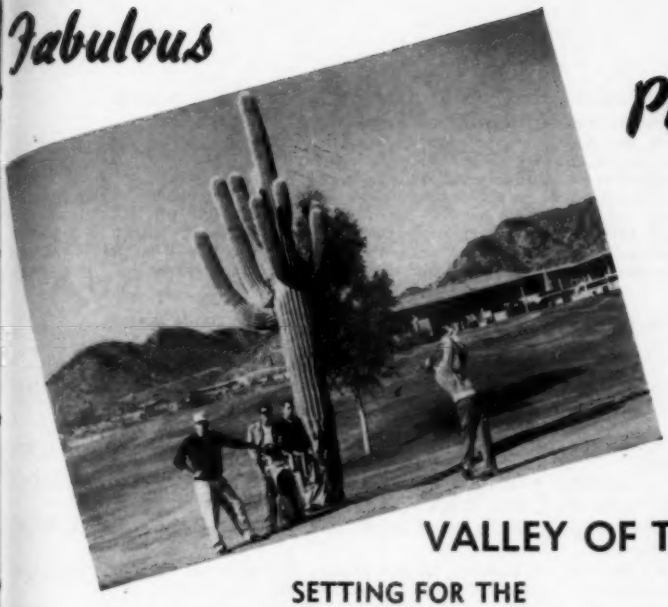

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ABSTRACTS

IMPROVEMENT IN PBI METHOD

By Hubert W. Marlow

Clinical Chemistry 4, 510, 1958 (December)

Varying quantities of pure crystalline to the $KClO_3$ were added to the 4N Na_2CO_3 used on the protein before drying in the Barker dry-ash method. The optional concentration was found to be 7.5 mg/ml. This eliminated the block insoluble residue after ashing so that centrifugation or clarification by filtration could be avoided. Incineration time at 600° can also be cut from 2½ hrs. to 2 hrs. with this modification. Data is given showing no adverse effect of the modification on the results.

E. FREIER

CHLORINE IN DISTILLED WATER AS A SOURCE OF LABORATORY ERROR

By Wendell T. Caraway

Clinical Chemistry 4, 513, 1958 (December)

Some distillation processes may result in the inadvertent contamination of distilled water with free chlorine. Free chlorine was found to have the following adverse effects:

1. An inhibitory effect on color development with four different uric acid methods.
2. Less azogilirubin formation when serum was diluted with chlorine containing water for the Evalyn & Malloy bilirubin method.
3. Decreased stability of the dilute iodide standards in the protein-bound Iodine procedure.
4. Slight effect on the phenol reaction step of the King-Armstrong method for phosphatase, oxyhemoglobin and methemoglobin procedures.
5. No effect on the Fiske-Subbarow phosphorus method; Benedict glucose method; bueret protein method; and ammonia nitrogen by nesslerization.

Tests for free chlorine in water are described.

E. FREIER

BOOK REVIEW

CHEMISTRY FOR MEDICAL TECHNOLGISTS

By Charles E. Selverd

C. V. Mosby Co., St. Louis, 1958. 465 pp. $10.75.

The paper is a fine grade. The type is legible; the cover is attractive. This is about the extent of faint praise that can be extended to this book. Approximately 136 pages (29% of the book) consists of directly quoted methods taken verbatim from the colorimeter manuals of the Photovolt, Leitz, Lumetron, or Coleman instruments. The average date of the publication of the methods selected for this volume is 1930, although the unwary might expect otherwise with the 1958 date on the volume itself.

The book is obviously intended for technicians of limited training, hence such unorthodox definitions as that of pH as "the exponent of the hydrogen ion concentration with the minus sign dropped". One could also easily argue with statements such as the one explaining the principle of photoelectric colorimeters. "The transmitted light is proportional to the depth of color which in turn is proportional to the concentration of the substance." An example of the dangers of this smattering of knowledge is in the chapter on Proteins. Directions are given for the Greenberg method for total protein, albumin and globulin using phenol reagent ending with "Determine concentration in gm. per 100 cc. of serum from the table." The next page is headed "Calibration of Colorimeter for Albumin and Globulin" and warns that all ensuing determinations must be performed by the procedure that follows. The procedure that follows is a Kjeldahl with Nesslerization rather than the Greenberg method. It is indeed unfortunate that the author used the term *Medical Technologist* in his title meanwhile using technician in the text. Medical Technology students in American Medical Association approved schools should be advised of this book lest the title mislead them.

ESTHER FREIER

SIMPLE RAPID TECHNIC FOR SERUM TOTAL CHOLESTEROL

MRS. EVELYN ABELL, MT (ASCP), *Diagnostic Clinic, Houston, Texas*

Determination of serum cholesterol is an important clinical evaluation. This paper presents a new and simplified procedure for the analysis of serum total cholesterol which affords maximum accuracy, speed and economy. In over 5000 determinations it has been found that reproducibility results are ±2%, with an average deviation of ±5%. Tables I and II will show the results of a limited number of these determinations.

The commonly used procedures of extraction [1-2] are eliminated and the technic is so simplified that 12 determinations can be made in 10 minutes. Sources of error are reduced to a minimum because only two reagents and the serum are pipetted. A small sample of serum is required; therefore, the method is ideal for children.

Among others, the laboratory at the Jefferson Davis Hospital had occasion to check the blood serum cholesterol method developed by H. A. Echols against the method of Zlatkis, Zak and Boyle (J. Lab. Clin. Med: 41, 486, 1953) which is normally employed. A B. & L. spectrophotometer 20 was the instrument used.

Standard Solutions gave the following results:

Table I
MULTIPLE DETERMINATIONS ON SAME SPECIMENS

Standard Value	DETERMINED VALUE BY METHOD OF:	
	Echols	Zlatkis, et al
50	48 (4)	51 (4)
100	103 (4)	101 (4)
150	151 (5)	153 (5)
200	202 (5)	198 (5)
250	250 (5)	247 (5)
300	297 (4)	301 (4)

Table II
DETERMINATIONS ON 18 HOSPITAL PATIENTS

METHOD		METHOD	
Echols	Zlatkis	Echols	Zlatkis
175	170	125	125
215	215	305	300
126	128	245	240
165	170	110	110
180	178	210	200
140	140	250	255
330	320	180	170
265	270	205	200
185	175	360	360

Acknowledgment

The author wishes to acknowledge gratefully the assistance of Dr. Joseph Elliott of the Jefferson Davis Hospital Laboratory in preparing the data for the above tables.

Reagents and Equipment

1. Echols Cholesterol Reagent
2. Sulfuric Acid—Concentrated, anhydrous—94.5—96.5%
3. Standard—Hyland's Clinical Chemistry Control Serum (or the equivalent) or any pooled serum of known analysis.
4. Colorimeter or Spectrometer having filter of 550-650 wavelength.

Procedure

Pipette into 125 ml. Erlenmeyer Flask:

(1) 4.6 ml. CHOLESTEROL REAGENT
(2) 0.9 ml. SULFURIC ACID
Mix laterally, immediately following addition of serum.
Allow to stand 3 minutes for complete reaction.
Transfer to absorption cell and read within 5 minutes, using 550-650 (640 preferred) wavelength.
Use reagent as blank.

Calculations:

$$\text{Factor} = F = \frac{\text{mg.\% cholesterol in standard}}{\text{Optical Density}}$$

$$\text{Mg.\% cholesterol in unknown} = F \times \text{O.D. unknown}$$

Observations

Synthetic cholesterol standards in glacial acetic acid or chloroform should not be used in this method. A "synthetic" standard is not recommended as the principle upon which this determination is based is that the speed of the complete reaction is dependent upon the heat created from water in serum when added to the reagent mixture. Reagents must be kept anhydrous and tightly capped when not in use.

CHOLESTEROL REAGENT must be stored at room temperature.

CHOLESTEROL REAGENT is known to be stable for 3 months. Normal and accelerated storage stability data indicate indefinite stability; however, until such data are available, laboratory stock in excess of a 3 months' requirement is not recommended.

COOLING of Reagent-Sulfuric Acid mixture (Step 2) is positively necessary for accurate results. It is not necessary to measure the exact temperature other than by touch. Cooling may be accomplished by:

(1) Allowing Reagent-Acid mixture to stand at room temperature and using the same day.
(2) Placing flask in pan of shallow water for short period of time, or
(3) Running water from tap over bottom of flask for a few seconds.

Your usual practice in good technique, in accurate volumetric transfers is essential to this method.

Summary

A simple rapid technic for evaluating Serum Total Cholesterol is described. The accuracy and reproducibility found in over 5000 evaluations is stated. The simplicity of the method insures minimum errors in the performance of the test.

References

1. Kenny, H. P.: The Determination of Cholesterol by the Liebermann-Burchard Reaction, Biochem. 5, 52: 611-619, 1952.
2. Zak, B., Dickenman, R. C., White, E. G., Burnett, H., and Cherney, P. J.: Rapid Estimation of Free and Total Cholesterol, Am. J. Clin. Path., 24: 1307-1315, 1954.